

ANNALES MEDICINAE EXPERIMENTALIS ET BIOLOGIAE FENNIAE

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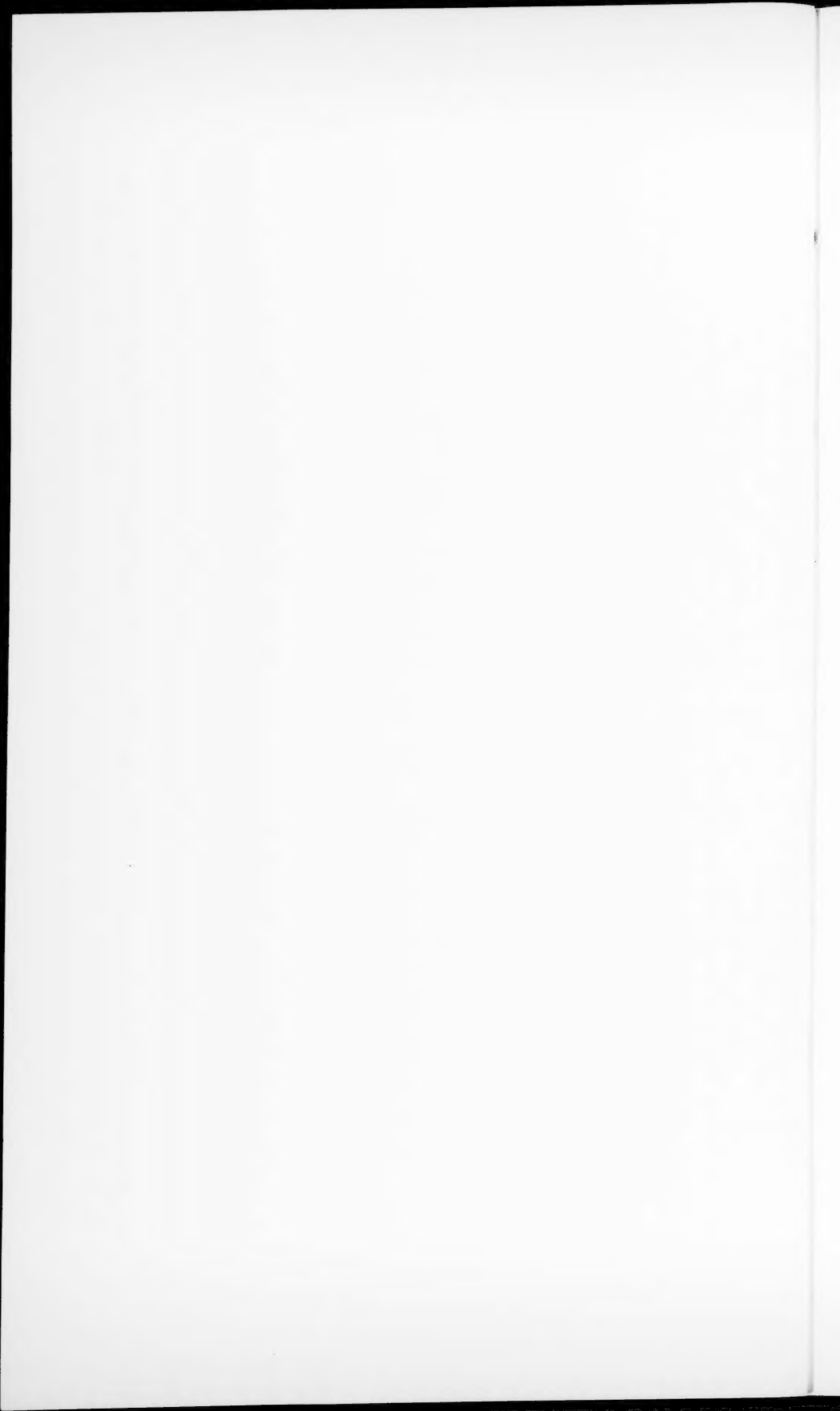
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OCCURRENCE OF *BACTERIUM ANITRATUM* IN
PATIENTS IN A CENTRAL HOSPITAL IN FINLAND

by

PAUL GRÖNROOS

(Received for publication September 13, 1958)

Bacterium anitratum (B.a.) is a gram-negative coccoid stab bacterium which does not reduce nitrate to nitrite. It was named B.a. by Schaub and Hauber in 1948 (14). Since that time the bacterium has been an object of increasing interest and, among its other properties, the taxonomic position and pathogenicity for humans have been investigated. From the available literature it seems probable that the same organism has been described under various names in several countries. Thus at least the *Diplococcus mucosus* described by von Lingelsheim in 1906 (9) and the *Herella vaginicola* of de Bord in 1939 (2) evidently are very closely related to B.a. The same may prove to be the case with the *Moraxella lwoffii* reported by Audreau (1). The definitive name of the organism will be determined by international agreement and probably will not be B.a. (15).

The pathogenic significance of B.a. for man is a subject of controversy. In the attempt to shed light upon this question I have during 2 ½ years systematically sought to isolate B.a. from samples sent to the laboratory of this hospital for bacterial culture. The hospital has 380 beds and is situated in Eastern Finland.

METHOD AND MATERIAL

As primary indicator of B.a. I used a 10 per cent lactose agar slant according to Schaub and Foley (13). This indicator proved

to be suitable; however, 6 strains of *Cloaca cloacae* were isolated which in the primary isolation were found to ferment 10 per cent but not 0.75 per cent lactose. Only one of these strains has retained this property. All the isolated strains of B.a. were capable of fermenting glucose (0.75 per cent) without the formation of gas. As a rule, acid was not formed in the Durham tube and the fluid in the tube therefore maintained its green colour. This phenomenon was of significance in the identification of B.a. A total of 62 strains were isolated in this manner. In the biochemical tests their behaviour was as follows:

Lactose (0.75 per cent), maltose, saccharose, mannite, dulcitol, adonitol, salicin and raffinose were not fermented.

The B.a. strains were capable of fermenting the following:

Arabinos:	in 24 hrs	29,	in 2—15 days	7,	negative	6
Rhamnos:	» » »	0,	» » »	» 14,	»	48
Galactose:	» » »	51,	» » »	» 11,	»	0
Xylose:	» » »	56,	» » »	» 6,	»	0
Production of urea:							
	in 24 hrs	2,	» » »	» 28,	»	28

All the strains were indole-negative, did not form H_2S in Sim (6) tubes, and were immobile. All the strains were nitrite negative after incubation in Tryptone (Difco) at $+37^\circ C$ for 24 hours. The strains were tested for urea both in a urea agar base according to Christensen (5) and in a modification of this base (6).

B.a. showed no growth on SS agar (Difco) and very weak growth on Staphylococcus No. 110 (Difco). Good growth, on the other hand, was observed on MacConcey agar, Summer citrate agar, Eosinmethylene blue agar, Levine EMB agar (Difco) and Tergitol No. 7 agar (Difco). On the last mentioned culture medium B.a. formed elevated colonies of an intense yellow colour. It probably is practically impossible to identify B.a. on the basis of type of colony growing on various culture media. The most frequently occurring type is arched, opaque and shiny, with even margins. A number of times there were seen on MacConcey agar small dark-red colonies superimposed on a pink colony growing from the smear proper. The small colonies were isolated and were found to differ in appearance from the »base colonies» which were re-isolated at the same

time. Both forms gave identical sugar series. Microscopically the bacteria were highly polymorphic, with no visible relationship to the type of the colony. Usually, however, there predominated a short, thick type of bacterium taking a bipolar stain and giving the impression of being a coccus. After a few passages long, frequently amorphous stabs were usually found among large masses of «cocci». These stabs were often present already after the first passage, while in some strains they were not seen even after two years. Certain strains were not always purely Gramme negative (compared with *Escherichia coli*).

A strain of B.a. selected at random was allowed to grow in an ordinary agar dish and then suspended in 5ml. of saline. This suspension, which was not subjected to any other treatment, was used for the production of rabbit immune serum. With various strains of B.a. the latter gave titres of 1/25—1/3200 (final) apparently without relationship to other observable properties. All the strains of B.a. reacted with undiluted immune serum, in addition to which, however, *Sarcina lutea* and certain other micrococci also produced agglutination. Strains of coli and staphylococcus were not agglutinated. B.a. strains boiled for two hours were not agglutinated. The serological method presented by Cary *et al.* (4) was not tested.

The strains were isolated from the following samples which had been sent to the laboratory for bacterial culture.

TABLE 1
SOURCE OF BACTERIUM ANITRATUM STRAINS

Source	No. of Strains
Ear	25
Urine	15
Faeces	8
Eczema secretion	6
Eye	5
Throat	2
Water from well	1

Simultaneously with B.a., one or more strains of other bacteria were isolated from the same samples, as is shown in Table 2. Faeces and throat samples were excluded, however.

TABLE 2
NUMBER OF STRAINS OF OTHER BACTERIA ISOLATED SIMULTANEOUSLY WITH
BACTERIUM ANITRATUM

0	1	2	3	4
10	23	10	4	3

B.a. was isolated simultaneously with the following bacteria:

<i>Staphylococcus aureus</i>	19 times
<i>Staphylococcus albus</i>	5 »
<i>Micrococcus saprophyticus</i>	9 »
<i>Diphtheroids</i>	8 »
<i>Candida albicans</i>	5 »
<i>Escherichia coli</i>	5 »
<i>Streptococcus alpha haemolyticus</i> ..	5 »
<i>Klebsiella</i>	5 »
<i>Proteus mirabilis</i>	2 »
<i>Faecalis alcaligenes</i>	2 »
<i>Pseudomonas aeruginosa</i>	2 »
<i>Escherichia freundii</i>	1 »
Group A streptococcus	1 »
<i>Pneumococcus</i>	1 »

In one case B.a. was isolated from well water containing 340 bacteria/ml. These, however, did not include *E. coli*.

The isolations of B.a. were almost equally distributed over the calendar months.

The patients from whom B.a. was isolated included 25 persons under 20 years of age, 9 of whom were less than 1 year old; 15 patients were aged 20—49 years and 21 were over 50 years.

Among the diagnoses with which the patients were dismissed from the hospital, the following occurred in more than one case:

Otitis media chronica	13	Cataracta senilis	4
Otitis externa chronica ..	6	Glaucoma	2
Ethmoiditis chronica	2	Gastroenteritis	3
Bronchopneumonia	2	Eczema acutum impetiginosa	2
Diabetes mellitus	2	Status post prostatectionem	2

TABLE 3

RESISTANCE AGAINST ANTIBIOTICS AND SULFA OF STRAINS OF STAPHYLOCOCCUS AUREUS ISOLATED FROM DIFFERENT SPECIMENS

Sensitivity	Sources of Isolation of the <i>Staphylococcus Aureus</i> Strains																			
	Pus, Eye, Urine, etc., except the Ear					From Nares of Hospital Staff					From Ears of Patients					Together with <i>Bacterium</i> <i>Anitratum</i>				
	S	P	Str	Tet	C	S	P	Str	Tet	C	S	P	Str	Tet	C	S	P	Str	Tet	C
0	40	40	22	36	0	46	44	20	32	2	40	40	6	24	2	14	22	1	7	3
1	6	14	14	10	6	2	4	4	10	14	10	4	2	6	8	4	1	7	7	8
2	10	16	12	26	40	0	0	6	4	20	6	4	7	16	30	3	1	4	8	10
3	14	34	40	40	40	0	0	18	2	12	6	18	40	22	28	4	0	12	2	3
A	57	38	26	32	0	96	92	42	67	4	64	60	10	35	3	54	92	4	29	12

Resistance of the strains against different antibiotics, determined by the method of Ericson *et al.* (7):

0 = resistant

1 = slightly sensitive

2 = moderately sensitive

3 = very sensitive

S = sulfa

P = penicillin

Str = streptomycin

Tet = tetracyclin

C = chloromycetin

A = per cent of resistant staphylococci

In addition there were 28 other diagnoses. With two exceptions these were manifested as more or less chronic conditions.

In one case B.a. was isolated from the faeces of twins aged 8 months, who had been treated with chloromycetin for one week. Both infants had gastroenteritis, and in addition to B.a. the faeces contained large numbers of *Candida albicans*. In a case of otitis media chronica, B.a. was isolated first from one year and ten days later from both ears. In a case (43 years, nephritis of the nephrones) B.a. was first obtained in association with *Cloaca cloacae* and 5 weeks later alone.

The staphylococci isolated simultaneously with strains of B.a. were penicillin resistant to an astonishing degree. The resistance of these strains against different antibiotics and a sulfa drug («Gantrisin») is shown in Table 3. For comparison there are included a number of strains of staphylococci isolated from other specimens.

DISCUSSION

The pathogenic significance of B.a. for humans is a subject of controversy. Pronounced virulence is regarded as rather rare. Moore (10) described a special form on conjunctivitis from which he had isolated B.a. (no detailed description of the strains was given). He stated nevertheless: »These facts would seem to indicate that the organism is moderately infective and that the human incubation period is in the region of 2 to 4 weeks». Waage (18) isolated B.a. together with *Staphylococcus aureus* from a patient with multiple abscesses of the brain. It is striking how often B.a. has been isolated with other bacteria or before or after the other bacterium was isolated from the same patient. *Proteus rettgeri* was isolated in one of the cases of B.a. bacteraemia reported by Rocha et Guze (12). The same authors were able to isolate 23 strains of B.a. during a period of 3 years. These authors as well as Brooke (3) stated that B.a. has been isolated together with other possible pathogens such as *Escherichia* and *Proteus*, and that only three times there was adequate evidence to establish a pathogenic rôle of B.a. In the series here presented, B.a. could not be demonstrated to have a pathogenic rôle in any of the cases. Philpot (11) found B.a. associated with organisms of the family *Micrococcaceae* in the urine of normal persons. He stated that the demonstration of this organism in the urine is not *per se* diagnostic of infection. The most generally accepted opinion appears to be that B.a. is of low virulence. This opinion is strongly supported by the series here presented. Subjects from whom B.a. could be isolated were patients with chronic diseases or of a reduced resistance due to other diseases. The series included a rather large number of children and old persons, and in general patients who had been treated with various antibiotics for some length of time. There was not a single patient who had not received penicillin before or at the same time as B.a. was isolated. Therefore it is not astonishing that the generally penicillin resistant B.a. can be isolated, together with other more or less saprophytic bacteria, from patients who had received penicillin therapy. This relationship is quite well reflected if we compare the resistance of the strains of *Staphylococcus aureus* isolated together with B.a., as is done in Table 3. The B.a. strains resemble quite markedly in their antibiotic resistance — except

for sulfa and streptomycin — the staphylococcus strains which were isolated from the hospital staff. The resistant staphylococci present an increasingly serious problem in hospitals. Occasionally they cause severe hospital epidemics or complicate individual cases. To this group of bacteria (*Staphylococcus aureus*, *Proteus*, *Pseudomonas*, etc.) which sometimes give rise to superimposed therapy-induced infections should apparently be added B.a. Usually B.a. occurs as purely a saprophyte in patients with a low primary resistance or with a bacterial flora whose natural equilibrium has been disturbed by intensive treatment with antibiotics.

SUMMARY

Sixty-one strains of *Bacterium anitratum* (B.a.) were isolated from patients in a Central Hospital in eastern Finland. A strain was isolated from contaminated well water. B.a. was generally isolated from patients with chronic diseases or with otherwise impaired resistance. It was often isolated with one or several other bacteria. Most frequently it occurred with *Staphylococcus aureus*. These staphylococcal strains were found to have a similar pattern of resistance to antibiotics as was seen in this hospital in strains isolated from the hospital staff. This, among other evidence, appears to indicate that B.a. infections belong to the hospital or therapy-induced type of infections. The author is of the opinion that B.a. is able to gain a foothold only in patients with impaired resistance or when the natural balance of the bacterial flora has been disturbed by intensive antibiotic therapy.

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RESPONSE OF RAT UTERINE MUSCLE TO N-METHYL- GLUCAMINATES OF MALE FERN EXTRACT AND] FLAVASPIDIC ACID¹

by

T. K. MARKKANEN, M. GRÖNROOS, and LEO HIRVONEN

(Received for publication September 6, 1959)

Vermifugal substances have a toxic effect on contractile cells. Both striated and smooth muscles are influenced by them.

The influence of male fern extract and flavaspidic acid on the heart muscle of rat has been reported in previous papers (2, 4).

The response of uterine muscle to male fern substances is of special interest during pregnancy. Since Kehrler (1) isolated uterine muscle preparations have been used to study the effect of many pharmacological substances.

The spontaneous contractile activity of an isolated rat uterine muscle preparation has been recorded under the influence of N-methyl-glucamines of male fern extract and flavaspidic acid.

METHOD

Thirty-three albino rats of the Wistar strain were used as experimental animals. Thirteen of those were normal fertile animals. Seven rats were ovariectomized one to six months earlier and thirteen were pregnant at the later part of pregnancy. The animal was stunned by a blow on the neck. A uterine muscle preparation of 1½ cm length was removed from the uterine horn above the uterine bifurcation. The serous membrane was removed with scissors from two sides of the preparation in order to promote the

¹ The writers are indebted to Pharmaceutical Manufacturers «Leiras» for economical aid.

muscular activity and the diffusion of the test substances. In pregnant animals the uterine preparation, which had a form of a tube, was cut with scissors to remove foetus and placental tissue. Due to this the preparations had a tape form.

The muscle preparation was connected with a thread with the bottom of an isolated organ bath and suspended with another thread from an isotonic lever. The ratio between the shorter lever arm and the longer one was 1: 5. The Tyrode solution used in the organ bath had following composition: Na Cl 8.0, KCl 0.2, CaCl_2 0.2, MgCl_2 0.1, NaH_2PO_4 0.05, NaHCO_3 1.0, glucose 1.0, and H_2O ad 1000.0. A continuous stream of 100 per cent oxygen was forced into the chamber through a sintered glass plate. A temperature of 32°C was maintained with a water bath. Generally the preparation showed immediate spontaneous contractions. The stabilization period before the beginning of the experiment was at least 15 minutes.

The vermifugal test substances, N-methylglucaminates of male fern extract and flavaspidic acid used in the present series were prepared as described previously (4). Only fresh solutions were used. A single dose of the respective substance was added to Tyrode solution surrounding the uterine muscle preparation. The mechanical function of the preparation was recorded on smoked paper. From these tracings it was possible to see the changes in the amplitude and rate of the contractions and in the tone of the uterine muscle. Each preparation was used only once.

RESULTS

Spontaneous activity of the uterine muscle preparation in pure Tyrode solution showed following characteristics. The contractions had a regular rate and even amplitude if the preparation was correctly made and its contractile activity stabilized (Fig. 1). The contraction period of the normal rat preparations was 146 ± 15 sec, which corresponds with a rate of 25 contractions per hr. The corresponding figures for the ovariectomized preparations were 73 ± 8 sec and 49 per hr and those of pregnant animals 114 ± 15 sec and 32 per hr.

High concentrations of test substances inhibited the contractions of the uterine preparations. In *contraction rate* a slight acceleration,

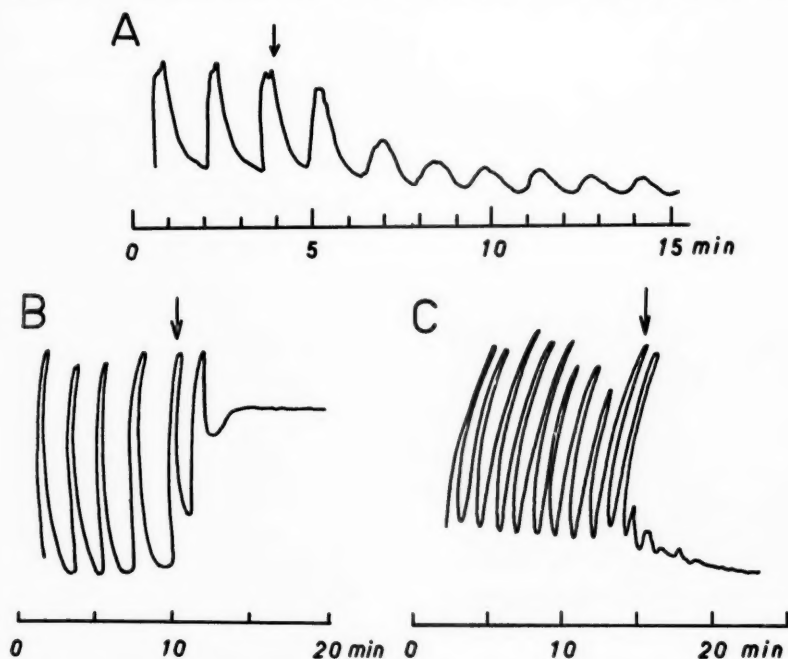


Fig. 1. — Response of isolated uterine muscle preparation of a non-pregnant (A), pregnant (B) and ovariectomized (C) rat to male fern extract glucamine in a concentration of 17 mg per 100 ml. The arrows indicate the administration of the test substance.

a retardation or no change took place during an observation period of 10 to 15 min. A transitory acceleration was probably more common in the preparations of pregnant rats than in those of other animals.

The *amplitude of contractions* decreased in all groups after addition of N-methylglucamines of both male fern extract and flavaspidic acid. The higher the concentration of the respective substance, the stronger was the diminution of the amplitude. No essential differences could be found between the effects of male fern extract and flavaspidic acid glucamines. Figs. 2 and 3 show the relative changes in the amplitude in non-pregnant animals. The absolute value for the amplitude before the administration of the test substance was 5 to 15 mm in various preparations.

The amplitude curves of pregnant rats are seen in Figs. 4 and 5. Some of these preparations were more resistant than those of non-

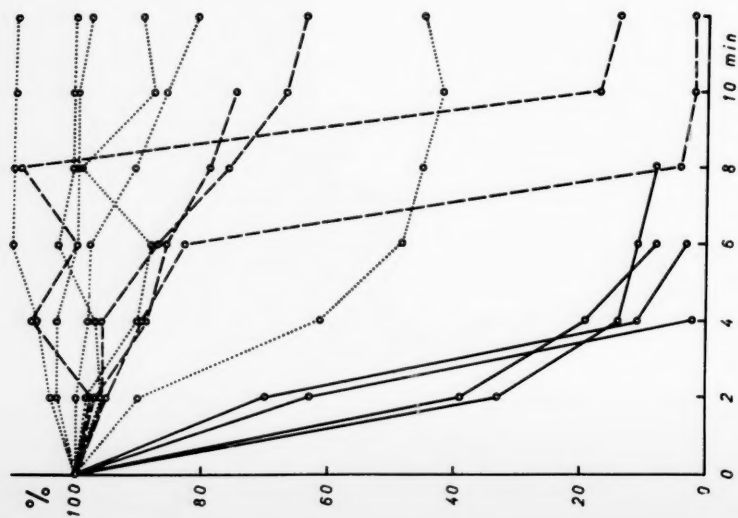


Fig. 2. — Relative changes in contraction amplitude of non-pregnant rat uterine preparations after addition of male fern extract glucuronate as function of time. Test substance concentrations: 0.17 mg per 100 ml, ——— 1.7 mg per 100 ml, ——— 17 mg per 100 ml.

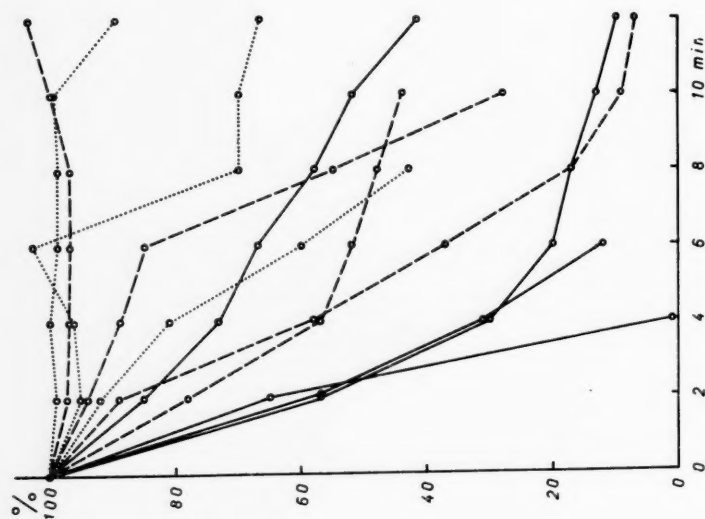


Fig. 3. — Relative changes in contraction amplitude of non-pregnant rat uterine preparations after addition of flavaspic acid glucuronate as function of time. Test substance concentrations: 0.17 mg per 100 ml, ——— 1.7 mg per 100 ml and ——— 17 mg per 100 ml.

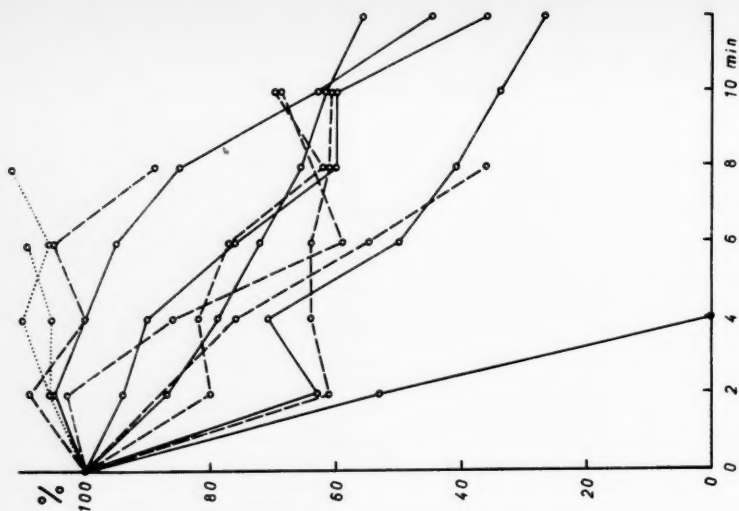


Fig. 4. — Relative contraction amplitude of uterine muscle preparations of pregnant rats after administration of male fern extract glucuronate in concentrations of 0.17 (.....), 1.7 (-----) and 17 (——) mg per 100 ml.

----- 1.7 mg per 100 ml, — 17 mg per 100 ml, 17 mg per 100 ml

----- 1.7 mg per 100 ml and — 17 mg per 100 ml

----- 1.7 mg per 100 ml and — 17 mg per 100 ml

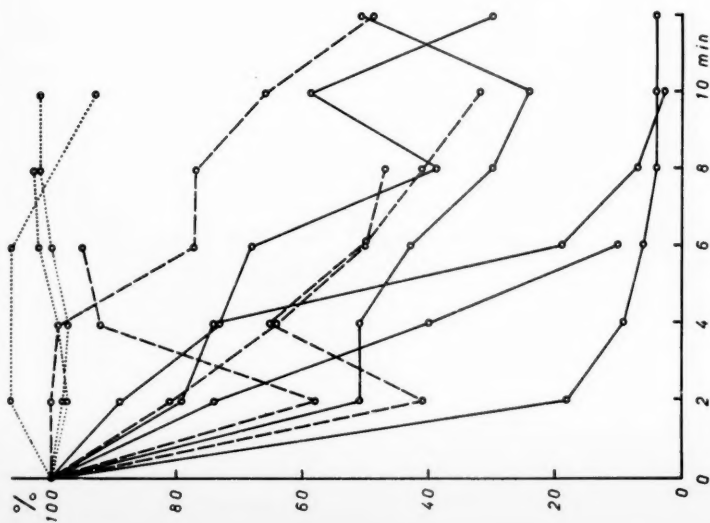


Fig. 5. — Amplitude changes of uterine preparations of pregnant rats after addition of flavaspidic acid glucamine in concentrations of 0.17 (.....), 1.7 (-----) and 17 (—) mg per 100 ml.

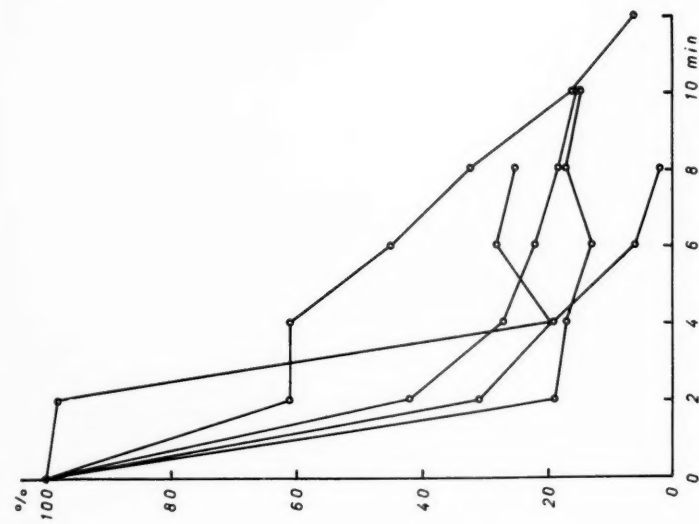


Fig. 6. — Relative diminution of contraction amplitude of uterine preparations from ovariectomized rats after addition of male fern extract glucamine in a concentration of 17 mg per 100 ml.

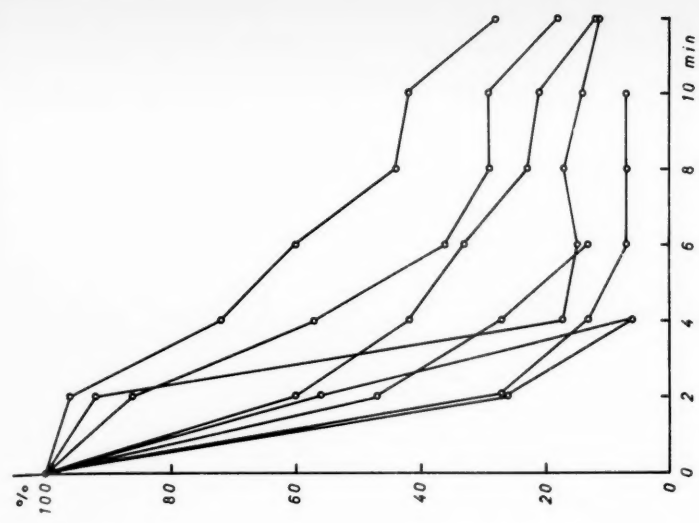


Fig. 7. — Response of contraction amplitude of uterine muscle preparations from ovariectomized rats in a flavaspidic acid glucamine concentration of 17 mg per 100 ml.

pregnant rats. Nothing can be said of possible differences between the influences of male fern extract and flavaspidic acid glucaminates.

The response of the uterine preparations from ovariectomized animals was similar to that of other rat groups (Figs. 6 and 7). Determinations were made only in one concentration.

The *muscular tone* of uterine preparations of non-pregnant and ovariectomized animals remained unchanged or decreased slightly under the influence of test substances (Figs. 1 A and 1 C). As for the uterine muscle of pregnant rats, there was a strong increase in tone in 3 cases of 9 preparations treated with male fern extract and in 5 of 9 preparations treated with flavaspidic acid (only concentrations of 17, and 17 mg per cent are included). A sample of this is seen in Fig. 1 B.

DISCUSSION

The contractile activity of the rat uterine muscle is regular if the preparation is carefully made (3); owing to this the rat uterus is suitable for this kind of experiments. The most important feature of the response of the rat uterus to male fern extract and flavaspidic acid glucaminates was a decrease in the amplitude of contractions. The higher the concentration of the respective substance, the greater was the diminution of the amplitude. No significant differences were found between the effects of male fern extract and flavaspidic acid. The resistance of the uterine muscle to test substances seems to be independent of the hormonal status of the animal as seen from the diminution of amplitude.

When the contractions were reduced to minimum, or totally inhibited, the tone of the uterine muscle decreased. Loss of tone is a characteristic effect of filicic acid on earth worm (6). In heart muscle preparations contracture has been observed (4). An interesting exception was found in uterine preparations of pregnant rats. An increase in the tone resulted in one third to one half of the experiments on pregnant animals. This may be due to hormonal factors. The importance of these factors is seen in the contraction rate of the various preparations. The rate was much higher in ovariectomized animals than in other cases. In all the various preparations the contraction rate decreased under influence of male fern extract and flavaspidic acid glucaminates.

The effect of vermifugal substances on uterine muscle is of interest because the combination helminthic parasites and pregnancy is not a rare one in areas where helminthic diseases are common. Male fern extract and flavaspidic acid has been found to increase foetal mortality in rats (5). Further investigations would be desirable to get information about conditions during pregnancy in woman.

SUMMARY

The effect of N-methylglucaminates of male fern extract and flavaspidic acid on rat uterus has been studied. The mechanical function of the isolated uterine muscle preparation was recorded isototonically in various concentrations of these substances. Fertile non-pregnant, pregnant and ovariectomized animals were used.

The test substances decreased the contraction amplitude and the contraction rate of the various preparations. No essential differences were found between the activities of the test substances. The uterine muscle of nonpregnant and ovariectomized rat was relaxed in high concentrations of male fern extract and flavaspidic acid glucaminates. In a part of the uterine preparations of pregnant animals an increase in tone was observed.

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THE VIRICIDAL ACTION OF ETHYLENE OXIDE

EXPERIENCE GAINED OF METHODS EMPLOYING TISSUE CULTURE FOR
CONTROL OF THE RESULTS

by

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(Received for publication September 17, 1959)

In recent years, ethylene oxide has been used more and more, especially for sterilization of thermolabile objects. Since 1928, this gas has been known to destroy microbes and its bactericidal effect in various circumstances has been studied in particular. On the other hand, but little is known of its effect on viruses. In the literature available, reports have been published from which the information in Table 1 has been taken. In the two first mentioned works (1—2) liquid ethylene oxide was used; its concentration was much higher than the gas suspension employed which contains 10 per cent ethylene oxide and 90 per cent carbon dioxide, as used in the present day to eliminate the danger of explosion. Klarenbeek and Tongeren have treated viruses dried on the surface of plastic tubes by normal gas pressure. Tissue cultures as a virus indicator were not made in their investigations.

As most of the known viruses multiply only in tissue cultures, the purpose of the present work was to study the action of ethylene oxide on the viruses growing in this medium. At the same time, endeavours were made to develop a method for removing the gas as quickly as possible from the samples treated with ethylene oxide before culture of virus in the tissue, because even a small quantity of gas destroys the tissue.

TABLE 1
EARLIER STUDIES ON THE VIRICIDAL ACTION OF ETHYLENE OXIDE

Author	Virus	Ethylene oxid	Temp.	Time after which no Propagation of Viruses was Noted (in Hours)	Virus-indicator
1. Wilson and Bruno 1950	Vaccinia	Liquid	37°C	24	Embryonated eggs
2. Ginsberg and Wilson 1950	Infl. A	Liquid	37°C	24	Embryonated eggs
	Infl. B			»	Embryonated eggs
	NCD			*	Embryonated eggs
	Columbia MM Theiler FA			* *	mouse mouse
3. Klarenbeek and Tongeren 1954	Vaccinia (dry)	Gas. Atmospher- ic. pressure	20°C	4	Embryonated eggs
	Columbia SK (dry)			8	mouse
4. Lammers and Gewalt 1958	Herpes	Gas. Pressure at 5—6 atm.	45°C	2/3	—
	Encephalomyo- caritis			*	—
	Theiler			*	—
	Phages			*	—

MATERIAL AND METHODS

In this study, herpes virus was chosen for the virus test as the changes caused by it in the tissue are easy to distinguish from other degenerate changes. The virus was a fresh strain removed from a patient; its infection titer was 10^{-3} . As the titer was not higher, a virus dilution of only 1:5 was used.

The viruses was propagated in tissue culture of the HeLa strain. The tissues were fed with a solution containing Parker's solution 90% and horse serum 10%.

The pre-tests showed that the HeLa tissue was rapidly destroyed by the gas dissolved in the cultur medium. Elimination of gas was possible in two ways: either by means of a neutralizing chemical procedure, or by the rapidity of gas evaporation. However, the chemical method could not be used as the chemicals used for neutralization, strong salt solutions or the thioglycate containing

the SH-series decrease the infections capacity of the virus or produces cytopathogenic changes in the culture cells. Moreover, it was not expedient to allow elimination of gas at its own speed as the time of cessation of its effect on the viruses would then not be revealed. Elimination of gas had to be accelerated. The best results were obtained by the samples treated with gas being put into a centrifuge (Spinco Ultracentrifuge Model L), in which evaporation of gas was done in vacuum. The evaporation surface was enlarged by making the centrifuge rotate 2000 times/min. When the quantity of liquid was 0.5 ml., the gas evaporated at the latest in 30 minutes but then it could not be avoided that, at the same time, also the fluid evaporated so that about 1/5—1/10 of the original quantity remained. Evidently the concentrated salts decrease the infectious capacity, as is seen from the subsequent results (0 hours control after vitalization). All of the remaining quantity was used for inoculation.

TABLE 2
EFFECT OF EXPOSURE TO ETHYLENE OXIDE. HERPES VIRUS TEMPERATURE
+ 45°C. PRESSURE 0.8 ATM.

Time in Hours		1	2	3	4	Control. No. Contact. (Tubes were sealed up)
0	No volatil- ization of medium.	+++	+++	+++	+++	
	After vola- tiliz.	++	++	+++	++	
1		++	++	++	++	++ +++ ++ ++
2		x	x	n	n	
3		x	n	n	xxx	++ +++ +++ ++
4		n	x	x	n	
5		xx	xx	x	xx	++ n ++ ++
10		x	xx	x	x	++ + ++ ++
Other controls:						
No virus. The medium exposed 10 h to gas						x x x x
Normals						n n n n

+++ just a few normal cells left

++ degenerated cells widely spread among many normal cells.

+ 2—3 foci of beginning cells degeneration

xxx } degenerate changes of a different degree not like those caused of
 xx } herpes virus
 x }
 n normal cells.

Gas treatment of the viruses was performed by a manufactured sterilization (Kaasukammio 4—43 Oy Santasalo-Sohlberg AB) device in which a gas pressure (10 per cent ethylene oxide and 90 per cent carbon dioxide) of 0.8 atm. and a heat of 20°C or 45°C was obtained. In the sterilization tests, the virus dilution was pipetted into a tube (10 × 100 mm) 0.5 ml. in each. The tubes were partly stopped with cottonpluggs, partly sealed up (control no contact, table 2 and 3) before putting in gas chamber.

TABLE 3

EFFECT OF EXPOSURE TO ETHYLENE OXIDE: HERPES-VIRUS TEMP. + 20°C.

Time in Hours		Tube				Control. No. Contact.			
		1	2	3	4	(Tubes were sealed up)			
0	No volatil- ization of medium.	+++	+++	+++	+++				
	After vola- tiliz.	++	++	+	++				
1									
2		—	++	+++	++	++	++	++	++
3		++	++	++	+++				
4		++	++	++	n	++	++	+++	++
5		++	++	++	xx				
6		xx	++	n	xx	++	++	n	++
7		n	xx	x	n				
8		xx	n	n	n	++	+	++	++
9		n	xx	xx	n				
10		n	xx	xx	x	++	+++	+	++
Other controls:									
No virus. The medium exposed 10 h to gas.						x	x	xx	x
Normals						n	n	n	n

After treatment with gas, the gas was evaporated as previously described, and the samples were inoculated for 72 hours in HeLa tissue.

In order to eliminate any possible subjective influence on the results, the tubes were marked with code numbers, which were not revealed to the investigators until the entire series had been studied. The count was made on the third, fourth, and fifth day after the period of incubation (in the tables the changes appearing correspond to the count on the fourth day).

RESULTS AND DISCUSSION

From Table 2 it is seen that at $+45^{\circ}\text{C}$ the virus loses its infectious capacity in 2 hours, and from Table 3 that at $+20^{\circ}\text{C}$ not until 7 hours after treatment. The subculture grown on the samples showed that it was still possible to culture virus from the tubes in which typical changes had been seen, but virus from the other tubes could not be cultured. As mentioned before, it is seen from the controls that evaporation of gas acted on the infectious capacity of the viruses. The samples which had not been treated with gas but evaporated in vacuum did not cause as large changes in the tissues as did the completely untreated samples. Thus the evaporation has contributed the effect of gas shortening the time of inactivation. We cannot obtain the absolute time of inactivation; we get mutually comparable results.

Lammers and Gewalt (4) have reported shorter periods (40 min.) for inactivation at $+45^{\circ}\text{C}$, but this was due to the use of considerably higher gas pressure (5–6 atm.). Moreover, they used hen embryo as a virus indicator.

Our results further confirm the well known fact that the use of tissue culture as virus indicator in disinfection tests is most unsatisfactory (5). Even small changes in the culture medium interfere with the tissue growth (changes marked x in the tables). It is inevitable that the changes be followed with code system and that subcultures of each sample be grown. This refers especially to viruses, as the changes caused by them in the tissues are difficult to distinguish from unspecific damages. Tests concerning adenovirus and poliovirus are in progress at the moment.

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MERCURIAL INHIBITION OF SUCCINIC DEHYDROGENASE AND SULFHYDRYL GROUPS IN RAT KIDNEY TUBULES WITH A NOTE ON THE EFFECT OF SOME NEW ORAL DIURETICS

by

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JYRKI RAEKALLIO

(Received for publication September 16, 1959)

In previous reports Mustakallio and Telkkä (7, 8) described the histochemical changes in succinic dehydrogenase activity of rat kidney tubules following the administration of six organomercurial diuretics and sublimate. Originally they employed blue tetrazolium (BT) for the demonstration of succinic dehydrogenase (7, 11). Later they used neotetrazolium (NT) which resulted in uniform staining patterns in contrast to the occasionally patchy BT-preparations (8). The use of iodonitrotetrazolium (INT) was no improvement since the coarse crystals of iodonitroformazan hampered identification of the different tubular segments (6). The histochemical methods for sulfhydryl groups revealed no reduction in sulfhydryls after administration of mercurials (8).

We have restudied the mercurial effects in rat kidney tubules with improved histochemical methods. In addition, some of the new oral diuretics have been included in this study.

METHODS AND RESULTS

We applied the recently developed nitro blue tetrazolium (Nitro-BT) method (9) for the demonstration of succinic dehydro-

genase because of its superior staining qualities. A further improvement of the technique was the use of a cryostat microtome adjusted to -24°C . It allowed to cut sections 5 microns in thickness.

The organomercurials mercaptomerin (Thiomerin «Wyeth»), Esidron «Ciba», and mercurophylline (Novurit «Medica») were subcutaneously administered in doses 7 to 30 mg Hg per kg of body weight to female Wistar albino rats weighing 150–200 g. After 24 hours the rats were killed by decapitation, and their kidneys were immediately processed.

Our results were essentially in accord with the previous reports (7, 8), *i.e.* the main site of inhibition by mercaptomerin was the middle part of proximal convoluted tubules, by Esidron the straight terminal portion of proximal convoluted tubules, and by mercurophylline the lowest part of the straight terminal portion of proximal convoluted tubules and the ascending thick limb of Henle's loops. The inhibition in the thick ascending limb was not so pronounced as revealed by BT which does not demonstrate a weak activity (8). Contrary to our findings, Wachstein and Meisel (13) reported no inhibition of succinic dehydrogenase in that segment of rat kidney after administration of mercurophylline.

The use of thin cryostat sections enables a more accurate identification of the different tubular segments. In addition, the dinitroformazan formed on reduction of Nitro-BT is practically insoluble in lipids and attaches directly to the mitochondrial proteins with elimination of the tendency to crystal formation in frozen sections (Fig. 1 and 2). Corresponding to the swelling of mitochondria induced by mercury, the stained particles were more coarse in the treated kidneys than in the controls.

A significant reduction in the sulfhydryl groups could be demonstrated when thin unfixed cryostat sections were treated with Bennett's sulfhydryl reagent (1) dissolved in dimethylformamide. The localization of the reduction of sulfhydryl groups corresponded well to the sites of succinic dehydrogenase inhibition (Fig. 3 and 4). This observation is in agreement with the results of Farah and associates (4).

The new oral diuretics chlorothiazide and dihydrochlorothiazide (Esidrex «Ciba») produced neither inhibition of succinic dehydrogenase nor reduction in sulfhydryl groups even in a subcutaneous dose of 1000 mg per kg of body weight. The chlorothiazides were



Fig. 1.

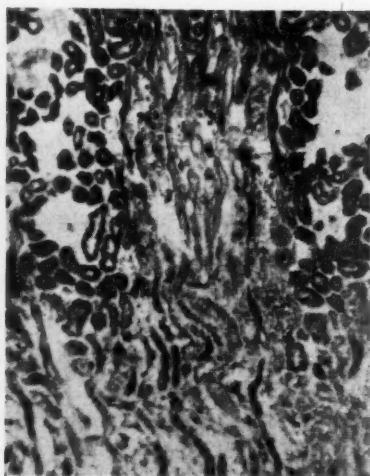


Fig. 2.



Fig. 3.

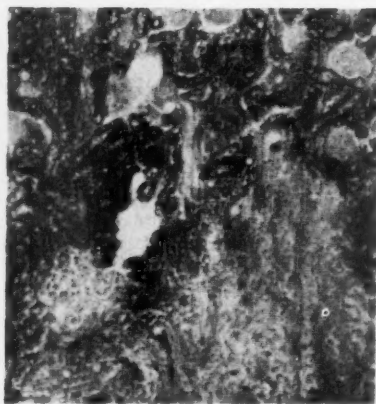


Fig. 4.

Fig. 1. — Succinic dehydrogenase activity in kidney cortex of a control rat. A cryostat section incubated with Nitro-BT. Note the amorphous deposition of dinitroformazan with good preservation of histochemical details.

Fig. 2. — Succinic dehydrogenase activity in kidney cortex of a rat killed 24 hours after administration of 15 mg of mercuriophylline-Hg per kg. Note the pronounced inhibition in the straight terminal portions of proximal convoluted tubules.

Fig. 3. — Sulfhydryl groups in kidney cortex of the same control rat as in Fig. 1. An unfixed cryostat section treated with Bennett's reagent dissolved in dimethylformamide. Note the monotonous distribution of sulfhydryl groups.

Fig. 4. — Sulfhydryl groups in kidney cortex of the same mercuriophylline rat as in Fig. 2. Reduction of sulfhydryl groups occurs at the corresponding sites as the inhibition of succinic dehydrogenase.

dissolved in a solution containing 80 per cent N, N'-dimethylacetamide and 20 per cent distilled water. The controls received a corresponding amount of the solvent.

Our attempt to localize the inhibition of carbonic anhydrase by sulfonamide diuretics failed since we could not reproduce the histochemical technique described by Kurata (5). Wachstein, too, was unsuccessful in demonstration of carbonic anhydrase activity by this method (12), whereas Braun-Falco and Rathjens (2) believed that the method is specific enough for the demonstration of carbonic anhydrase. A recent reappraisal of the method revealed that it does not stain carbonic anhydrase. The procedure seems to result in the precipitation of divalent cation carbonates more insoluble than cobaltous carbonate. These cations are responsible for the localization of staining (3).

SUMMARY

Nitro blue tetrazolium was found to be superior to the other tetrazolium salts in the localization of mercurial inhibition of succinic dehydrogenase in kidney tubules of rat.

The reduction of sulfhydryl groups induced by organomercurial diuretics could be demonstrated in thin cryostat sections with a modification of Bennett's technique. Both succinic dehydrogenase inhibition and reduction in sulfhydryl groups occurred at the corresponding sites.

Inhibition of succinic dehydrogenase and sulfhydryl groups by chlorothiazide and dihydrochlorothiazide was not demonstrable by histochemical means. The histochemical demonstration of carbonic anhydrase inhibition by sulfonamide diuretics must wait for the development of a reliable method.

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THE DEVELOPMENT OF HEMAGGLUTININS IN THE SEEDS OF VICIA CRACCA

by

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The agglutinins in ripe seeds will frequently be missed in unripe ones. Their absence in the young seeds of *Vicia Cracca* is mentioned by Krüpe (1). The late appearance of agglutinins must be connected with the process of ripening. Interested in this phenomenon we made the following experiments:

TABLE 1
SALINE EXTRACTS (1/20) OF GREEN SEEDS OF VICIA CRACCA (LOT 1) TITRATED
AGAINST A1 CELLS

The Size of the Seeds in mg	Extract Dilutions										
	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	NaCl
>27	+++	+++	+++	+++	+++	+++	++	+++	++	—?	—
24—26	++	+++	+++	+++	+++	+	—	—	—	—	
21—23	—	—	—	—	—	—	—	—	—	—	
18—20	—	—	—?	+++	++	—?	—	—	—	—	
15—17	—	—	—	—	—	—	—	—	—	—	
12—14	—	—	—	—	—	—	—	—	—	—	
12 <	—	—	—	—	—	—	—	—	—	—	

Apparently, at a certain stage the agglutinins appeared rather suddenly. Inasmuch as all the green seeds contained considerable amounts of water, the size of seeds is presumably still at this stage correlated to their age. We obtained further information of the

¹ Aided by a grant from Sigrid Jusélius Foundation.

maturation of seeds from a germination experiment. The day the seeds were collected they were put between wet sheets of cellulose and then observed during three weeks.

TABLE 2

RELATIVE GERMINATION RATE OF GREEN, FRESH SEEDS OF VICIA CRACCA (LOT 1)

Size of the Seeds	Seeds Germinated
27	24/30 i.e. 80 per cent
21—26	13/30 40
15—20	1/21 5

The germination rate seems to increase with the size of the seeds and coincide with the appearance of agglutinins in them (Table 1).

The absence of agglutinins in small green seeds, where they weren't found even by albumin technic arises the question of possible inhibitors. The following experiments may throw light upon the problem:

TABLE 3

INHIBITION OF NORMAL VICIA CRACCA AGGLUTININS BY SALINE EXTRACTS (1/20) OF THE GREEN SEEDS OF THE SAME SPECIES (LOT 2).

After pipetting of the agglutinins and inhibitors, the presence of agglutinins was successively tested with A1 cells.

Inhibitors Tested:	Size of the Seeds	Dilutions of a Saline Extract 1/20 of Ripe Seeds of Vicia Cracca									
		1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	NaCl
Saline extracts of green seeds	>24 mg	+++	+++	++	++	—	—	—	—	—	—
of Vicia Cracca	10—15 mg	(+)	—	—	—	—	—	—	—	—	—
Saline		+++	+++	+++	+++	+++	+++	±	±	—	—

There is but little doubt that the extract of the green seeds contained inhibitors against the agglutinins of the ripe seeds of the same species.

The amount of inhibitors in the green seeds of Vicia Cracca is correlated to the size of the seeds in Table 4.

Here the amount of inhibitors did not vary very much. However, it is possible that there is first a slow increase and then a slow decrease in the amount of inhibitor during the development of the seeds.

THE INHIBITION OF NORMAL AGGLUTININS OF VICIA CRACCA BY SALINE EXTRACTS (1/20) OF FRESH GREEN SEEDS OF THE SAME PLANT (LOT 2) A1 CELLS. FOUR-FOULD THE MINIMUM ACTIVE AMOUNT OF AGGLUTININS.

TABLE 5

SALINE EXTRACTS 1/20 OF DRIED, GREEN SEEDS OF Vicia cracca (LOT 1)
TITRATED AGAINST A1 CELLS

[illegible]

Comparing the Table 1 with Table 5 it is evident, that the agglutinins appeared still more suddenly in the extracts of dried green seeds, than in the extracts of fresh seeds. Especially, it is noteworthy that the extract of seeds, size 21—23 mg, did not contain agglutinins when fresh seeds were extracted (Table 1) but contained them after the seeds had been dried (Table 5). By drying, we know, in the small seeds the inhibitors remain unchanged. However, in this particular group of seeds the inhibitor was destroyed during the drying.

SUMMARY

The saline extract of young green seeds of *Vicia Cracca* inhibit the normal agglutinins of the plant. During the development of the seeds the amount of the inhibitor might slowly increase and then gradually decrease. Then all of a sudden the agglutinins appear. The critical stage of development of agglutinins coincides with the critical stage of seeds where the capacity to germinate appears.

The inhibitor in the green seeds of *Vicia Cracca* is rather thermostable. It is not precipitable by acetone like the agglutinins. Thus, there are points of likeness with the carbohydrates known to inhibit the hemagglutinins in seeds. The inhibitor in *Vicia Cracca* is unspecific, inhibiting, more or less agglutinins of other seeds too, but it is very weak against the human isoagglutinins.

At a certain critical stage the agglutinins will appear and inhibitors disappear but after the seeds are dried.

The green seeds of *Vicia villosa* contain a similar inhibitor.

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REDUCING THE TOXICITY OF PENICILLIN FOR GUINEA PIGS

by

W. J. KAIPAINEN

(Received for publication September 28, 1959)

The toxicity of penicillin for guinea pigs, first studied by Hamre *et al.* (1), is an established fact. It is extraordinary that the guinea pig tolerates poorly especially penicillin which is one of the only antibiotics in extensive use the dosage of which can be stepped up to gigantic proportions for human subjects without side effects. Many explanations have been proffered concerning the toxicity of penicillin for guinea pigs, but none have found general acceptance. Attempts have also been made to overcome the toxic effects of penicillin in guinea pigs, but without success.

Veterinary medicine has for some time used a dried, autoclave-sterilised extract made from the contents of cow rumen. This extract has helped neat cattle suffering from loss of appetite after antibiotic therapy. The daily administration of 250 g of rumen extract restores the appetite and rapidly revives the animals. A penicillin injection affects the guinea pigs with loss of appetite in approx. 3 days and they begin to lose weight. Diarrhea occurs concurrently and the majority of the guinea pigs die in the course of 2—3 weeks. The loss-of-appetite symptoms resemble so closely the symptoms observed in calves that it was decided to experiment with the rumen juice on guinea pigs suffering from toxicity symptoms caused by penicillin.

MATERIAL

The rumen juice was provided by the Danish Biofac company. It was prepared by recovering the contents of the rumen immediately after killing the animal at the slaughter-house. The contents were then frozen, extracted and sterilised in an autoclave. 50 per cent of potato flour was added to facilitate handling. A fresh water mixture of the preparation was made daily and fed to the guinea pigs orally through a tube.

The penicillin preparation used was crystalline penicillin G (Benzyl penicillin sodium Leo), administered in an aqueous solution to the guinea pigs intraperitoneally on two successive days,

TABLE 1

THE TOXICITY OF PENICILLIN FOR GUINEA PIGS TREATED BY DIFFERENT METHODS. 100,000 I.U. OF PENICILLIN WAS ADMINISTERED INTRAPERITONEALLY ON TWO SUCCESSIVE DAYS.

No. of Animals	Weight	Treatment	Survivors after			
			1st	2nd	4th	8th week
<i>Rumen extract</i>						
5	850—600	1 g twice daily from the 1st day	5	5	4	4
5	1100—850	100 mg twice daily from the 1st day	5	4	2	1
5	570—400	10 mg twice daily from the 1st day	5	1	0	0
5	920—780	1 g twice daily from the 6th day	5	5	3	3
<i>Penicillinase</i>						
5	900—780	Intraperitoneally on the 1st and 2nd day				
		15 minutes after administration of penicillin	2	0	0	0
5	790—740	Intraperitoneally on the 6th and 7th day	5	2	0	0
<i>Potato flour</i>						
5	1120—720	0.5 g twice daily from the 1st day	2	0	0	0
5	560—390	No treatment	3	0	0	0
5	910—600	No treatment	4	2	0	0

a daily dose of 100,000 units. The effect of penicillinase was also tested. The preparation available was Sterile penicillinase D.C. (B.)L. 1 ml, diluted according to instructions, inactivated no less than 1,000,000 I.U. of penicillin. 0.5 ml of this preparation was administered on two successive days. Hence a dose that would have sufficed to inactivate 1,000,000 I.U. was used in this case to inactivate 200,000 I.U. of penicillin.

RESULTS

Table 1 shows the number, weight, medication and number of survivors during 8 weeks. In the control series, to which no medication was given, the guinea pigs generally died within 2—3 weeks of the administration of penicillin. All guinea pigs receiving penicillinase also died. Penicillinase administered 15 minutes after the injection of penicillin was thus incapable of inactivating its effect. In the group given 100 mg of rumen juice twice daily not all the guinea pigs had died after 4 weeks. One g of rumen juice was given twice a day on the sixth and seventh day after the penicillin dose to 5 guinea pigs; 3 were still alive 8 weeks later. When the administration of the rumen juice was commenced simultaneously with penicillin, 4 out of 5 guinea pigs remained alive for the 8 weeks of the experiment. For control purposes, a group of guinea pigs was given potato flour only in lieu of the rumen juice; all of them died within 2 weeks.

Table 2 gives the results of the weekly weight readings of the three groups given rumen juice and the control group. The weight drop after 1 week was percentually nearly the same in each group, continuing in the second week on the same scale in all the groups. In the group given rumen juice simultaneously with penicillin the weights increased perceptibly although the rumen therapy was terminated at the end of the third week. In the group in which rumen therapy was started on the sixth day from the administration of penicillin the weight of the surviving guinea pigs was followed after the fourth week. They were found to have regained their original weight within two months of the penicillin injection. It was stated in an earlier investigation (2) that guinea pigs surviving after one injection of 100,000 I.U. of penicillin regained their original weight first in c. 6 months.

TABLE 2

THE EFFECT OF RUMEN EXTRACT ON THE WEIGHT OF GUINEA PIGS SUFFERING FROM INTOXICATION SYMPTOMS CAUSED BY PENICILLIN.

Treatment	Number of Animal	Original Weight	After 1 Week	After 2 Weeks	After 4 Weeks
Rumen extract 1 g twice a day, from the 1st day	1	700	500	450	530
	2	850	630	560	600
	3	750	550	610	670
	4	600	460	400	600
	5	600	450	340	—
1 g twice a day, from the 6th day	6	780	660	560	590
	7	920	740	600	—
	8	850	670	580	740
	9	870	720	600	—
	10	900	760	660	740
0.1 g twice a day, from the 1st day	11	1000	870	—	—
	12	1100	810	690	660
	13	1100	900	800	630
	14	850	620	480	—
	15	1000	800	690	650
No treatment	16	820	700	580	—
	17	860	700	—	—
	18	600	440	—	—
	19	910	760	740	—
	20	900	—	—	—

DISCUSSION

Forced feeding of an extract of cow rumen juice was found to diminish the toxicity of penicillin for guinea pigs. It has not yet been established whether this effect is due to the forced feeding of the juice, *i.e.* whether it is just a matter of a substitute for the food the guinea pigs are unwilling to eat, or whether some substance(s) present in the rumen juice is involved, something influencing the mechanism disturbed by the penicillin. The weight loss in both the rumen extract group and the control group (no rumen extract) was of the same magnitude during the first and the second weeks. The animals of the control group died whereas the animals given rumen juice gradually revived and by the third week had begun to eat relatively well. Hence forced feeding with rumen juice obviously had no direct effect on the weight, at least not

during the first two weeks. Moreover, as nutrition, the amount of rumen extract and potato flour was relatively small (maximum 2 g per day).

When rumen extract was used on calves the doses administered were much smaller. It has been suggested that the beneficial effect of rumen extract in cattle lies in its action on the intestinal bacterial flora. Its effect mechanism in calves, however, is still being studied.

SUMMARY

It was possible to counter the toxic effect on guinea pigs of 100,000 I.U. of penicillin administered intraperitoneally on two successive days if the animals were simultaneously force-fed daily with 1 g of rumen extract. This preparation was obtained from the contents of the rumen of slaughtered cows. The autoclave-sterilised rumen extract was unable to prevent a weight loss in guinea pigs in the first and second week after the injection of penicillin. The guinea pigs in the control group and those treated with penicillinase died within 2—3 weeks. But guinea pigs given the extract gradually began in the third week to eat voluntarily and regained lost weight in approx. two months.

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NUCLEOTIDES AND AMINO ACIDS OF THE LATHYRITIC CHICK EMBRYOS

by

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(Received for publication October 2, 1959)

This study was motivated by the morphological changes in the connective tissue cells in lathyrism (1, 4). Other studies had suggested an effect on the nucleotides of the muscle (5) and implicated the energy metabolism by increased respiration both in lathyrism *in vivo* (6) and in tissue slices incubated with β -aminopropionitrile (3). We also liked to use the opportunity to check our earlier claims on amino acids and peptides in the muscle of lathyritic rats (5).

EXPERIMENTAL

Embryos. — Eggs (about 20—30 in each group) were hatched at $+37^{\circ}\text{C}$. temperature and 75% relative humidity. On the eighth day 0.314 mg. of β -aminopropionitrile (as sulfate dissolved into 0.075 ml. of distilled water) was injected into the yolk sac. The same volume of distilled water was injected into the control eggs. Three sets of experiments were made.

Nucleotides. — On the twelfth day the embryos (which showed several malformations) were homogenized into 0.6 *N* perchloric acid in a Potter-Elvehjem homogenizer. The supernatant was obtained by centrifugation and neutralized with 5 *N* potassium hydroxide. The new supernatant was fractionated in Dowex-1 column using gradient elution with formic acid and ammonium formate according to Hurlbert *et al.* (2). The extinctions at 2600 Å were measured with Beckman DU spectrophotometer. Figure 1 shows a typical elution diagram. In the first experiment (shown in the figure) a difference was found between the experimental and control

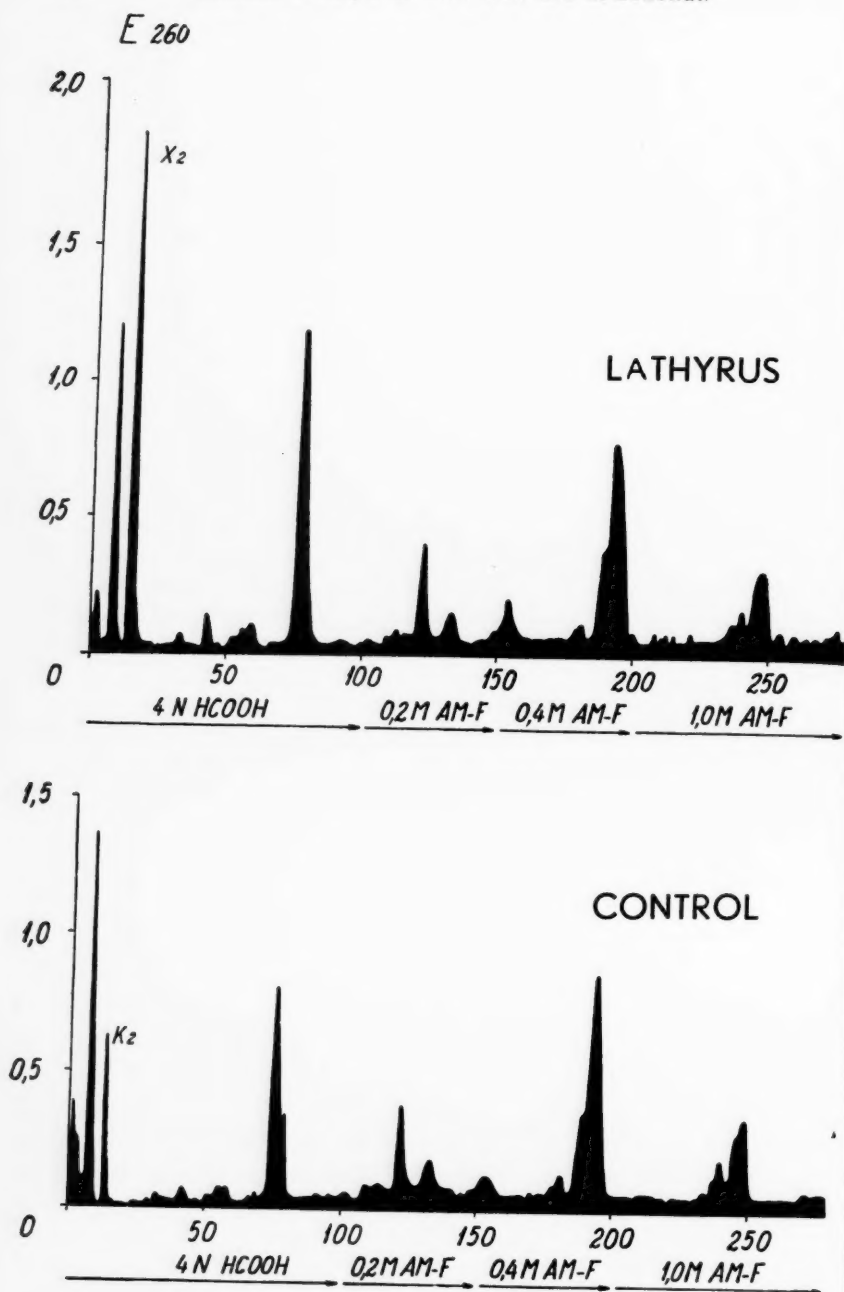


Fig. 1. — The elution diagrams of nucleotides from the lathyrotic (upper curve) and control (lower curve) embryos. Abscissa: 1 ml. fractions, AM-F = ammonium formate.

sample. The peak marked with X_2 was relatively much higher than the corresponding control peak K_2 . The peaks K_2 and X_2 were analyzed by paper chromatography and by spectrophotometry and found to be nearly pure AMP. So in this experiment AMP was relatively increased in lathyritic embryos. However, in later experiments this could not be confirmed.

Amino Acids. — Homogenate (in distilled water) was precipitated with ethanol (final conc. 80% v/v). The supernatant was evaporated at boiling water bath and the residue dissolved in acetone containing 1% of conc. hydrochloric acid (v/v). Two-dimensional chromatograms were prepared using both butanol-acetic acid-water (4:1:5) and water-saturated phenol (containing 1% conc. ammonia solution v/v) as solvents. The chromatograms were also repeated after hydrolysis in 6 *N* hydrochloric acid at +100°C. for 20 hr. in closed tubes. No differences were observed and the results are not presented in detail.

RESULTS AND DISCUSSION

These experiments do not confirm the idea that nucleotide metabolism would be primarily affected in lathyrism. Some support to this negative conclusion is also obtained from unpublished data on incorporation of ^{15}N -marked ammonia into purine, which was not altered in lathyritic chick embryos. The findings described earlier in the muscle seem to be due to secondary effects, for example, inanition or atrophy. The same explanation applies to amino acid composition.

SUMMARY

No changes were observed in the free nucleotides or amino acids in the lathyritic chick embryos.

Acknowledgements. — Messrs. Turun Muna, Turku, Finland, provided as a gift the fertilized eggs. The unpublished data on ^{15}N incorporation were obtained by Dr. H. Land, Institute of Biochemistry, Helsinki. »Sigrid Jusélius Foundation» and State Board of Science have supported this program financially.

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HERZFREQUENZVERÄNDERUNGEN BEI SCHNELLEN TEMPERATURSPRÜNGEN

von

KURT BOMAN

(Bei der Schriftleitung eingegangen am 4. Oktober, 1959)

Die Abhängigkeit der Schlagfrequenz des Herzens von der Schrittmachertemperatur wurde zuerst von Bowditch (3) beobachtet. Seit der Zeit sind mehrere Untersuchungen über dieses Gebiet veröffentlicht worden, es konnte allerdings nicht mit Sicherheit festgestellt werden, ob die Beziehung zwischen der Temperatur und der Schlagfrequenz eine lineare oder eine logarithmische ist (4, 5, 9). In neuester Zeit wird allgemein das lineare Verhältnis anerkannt, jedenfalls zwischen 3 und 22 °C im Froschherzen (6) und im Warmblüterherzen zwischen 25 und 38 °C (1).

Bei der Untersuchung über die thermosensiblen spontantätigen Rezeptoren z.B. der Warmblüterzunge (8), der Lorenzinischen Ampullen (7) und der Haut (2) wurde beobachtet, dass eine gewisse, von der Grösse des Reiztemperatursprunges abhängige Einstellzeit notwendig ist, bevor eine konstante Entladung bei einer konstanten Reiztemperatur erreicht wird. So ergab sich u.a., dass die Thermo-rezeptoren der Haut bei Änderung der Hauttemperatur eine überschüssende Erregung bzw. Hemmung während des Temperatursprunges aufweisen. Es schien uns daher wissenswert, ob auch die Spontanrhythmik der Schrittmacherfasern des Kaltblüterherzens ein ähnliches Verhalten zur Temperatur zeigen.

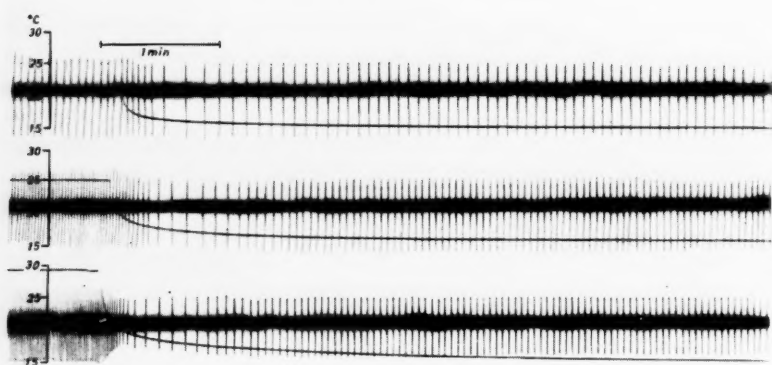


Abb. 1. — Aktionspotentiale eines Froschvorhofpräparates bei Kältesprüngen von 21, 25 und 29 °C auf 15 °C.

Als Untersuchungsobjekte wurden spontanschlagende Froschvorhofpräparate verwandt. Die mit einem Circulärschnitt entfernten Vorhöfe wurden mit Sauerstoff in Ringerlösung durchgespült, für die Sauerstoffmangelversuche wurde die Sauerstoffzufuhr gedrosselt und Stickstoff wurde eingeführt. Die Registrierung der Vorhofstemperaturen wurde thermoelektrisch durchgeführt, das Thermoelement wurde vorsichtig in den Vorhof eingeführt und die erzeugten Thermoströme wurden in üblicher Weise durch einen Galvanometer in die Registrierkamera übergeleitet. Das Präparat wurde auf eine Thermode gelegt, deren Temperatur beliebig variiert werden konnte. Die Aktionsströme wurden mit Platinoberflächenelektroden abgeleitet und auf dem Kathodenoscillografenschirm aufgezeichnet. Die Registrierung erfolgte mit einer Registrierkamera, wobei die gleichzeitige Registrierung der Aktionsströme und der Vorhofstemperatur möglich war.

ERGEBNISSE

Abb. 1. zeigt das typische Verhalten der Schlagfrequenz eines Vorhofes bei schneller Abkühlung. Das Diagramm in der Abb. 2 stellt die Schlagfrequenz dieser Präparation als Funktion der

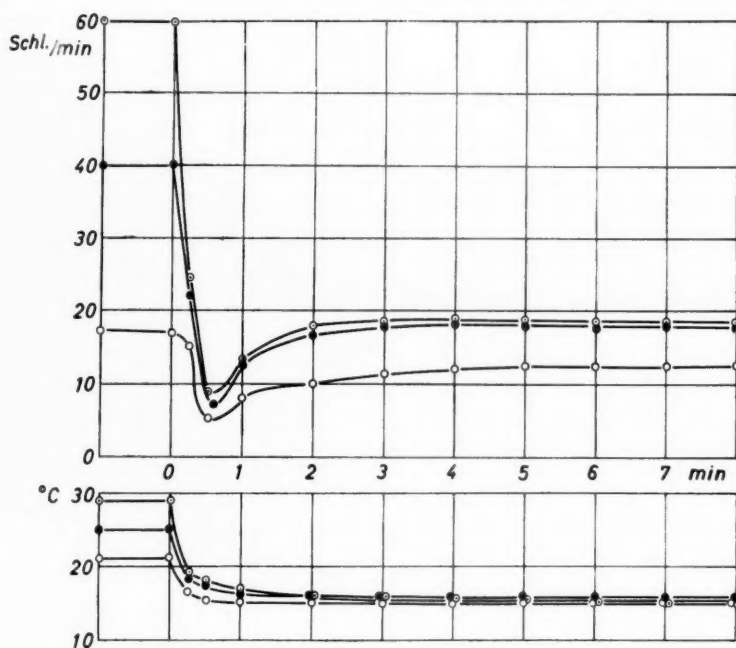


Abb. 2. — Zeitlicher Verlauf der Schlagfrequenz und der Vorhofstemperatur bei Kältesprüngen von 21, 25 und 29 °C auf 15 °C.

Zeit dar. Der Vorhof war jeweils 15 min. auf die Ausgangstemperatur adaptiert, die Sauerstoffzufuhr war während dieser Zeit gedrosselt. Die Temperatursprünge betrugen von oben nach unten 6, 10, und 14°C. Die grösste Temperaturänderungsgeschwindigkeit war 5°/sec. Die allmählich eintretende überschüssige Hemmung ist deutlich ersichtlich. Die grösste Verlangsamung der Schlagfrequenz tritt nach 30 sec. ein und stellt sich wieder auf die konstante Reiztemperatur in etwa 2 bis 3 min. ein. Die maximale Abnahme der Schlagfrequenz als Funktion der Grösse des Kältesprungs ist ziemlich gradlinig.

Abb. 3 stellt das Verhalten eines Vorhofes bei Wärmesprüngen verschiedener Grösse dar. Die Abb. 4 zeigt die Schlagfrequenz dieses Präparates als Funktion der Zeit. Die Versuchsverhältnisse waren wie in dem ersten Versuche. Ganz im Gegensatz zur Abkühlung wird hier eine überschüssige Erregung beobachtet. Die Wärmesprünge betrugen 3, 7, 5 und 12°C. Die deutlichste

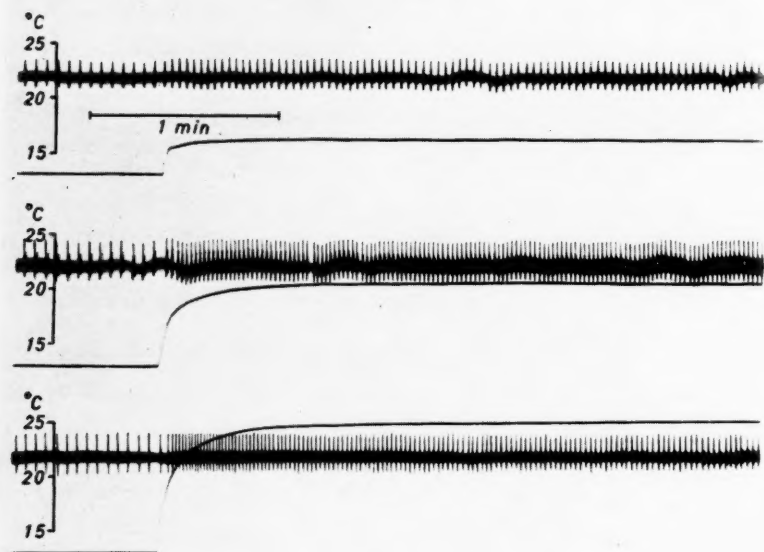


Abb. 3. — Aktionspotentiale eines Froschvorhofpräparates bei Wärmesprüngen von 13 °C auf 16, 20.5 und 25 °C.

Zunahme wird hier erst 30 sec. nach Beginn des Wärmesprunges beobachtet. Die Einstellung auf die konstante Temperatur erfolgt auch hier in 2 bis 3 min. Die maximale Zunahme der Tätigkeit ist deutlich von der Grösse des Wärmesprunges abhängig.

BESPRECHUNG DER ERGEBNISSE

Die oben erwähnten Beispiele stellen das Verhalten der Schlagfrequenz des Froschvorhofes bei Temperatursprüngen dar. Allerdings muss in Betracht genommen werden, dass die typischen Erscheinungen der überschüssenden Tätigkeit meistens nur bei grösseren Temperatursprüngen mit erheblicher Temperaturänderungsgeschwindigkeit zum Vorschein kommen. Die Einstellung auf die konstante, der Temperatur entsprechenden Schlagfrequenz erfolgt bei kleinen oder langsamen Temperatursprüngen meistens ohne deutliche überschüssende Erregung oder Hemmung. Diese Erscheinung scheint auch sehr abhängig zu sein von der Ermüdung

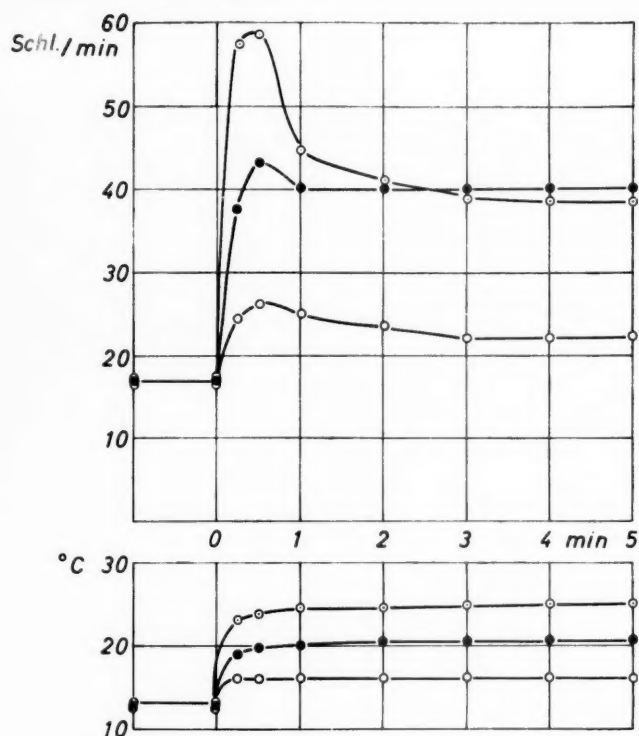


Abb. 4. — Zeitlicher Verlauf der Schlagfrequenz und der Vorhofstemperatur bei Kältesprüngen von 13 °C auf 16, 20.5 und 25 °C.

und Sauerstoffversorgung des Vorhofes. In älteren Präparationen und bei Sauerstoffmangel wird die überschüssige Tätigkeit am Anfang des Temperatursprunges besonders deutlich beobachtet, wogegen dieselbe bei jüngeren, frischen Präparaten manchmal undeutlicher ist.

So wie bei den anderen spontantätigen nervösen Strukturen handelt es sich auch in den Schrittmacherfasern des Kaltblüterherzens um Vorgänge in erster Linie chemischer Art, die durch Temperaturen steuerbar sind. Der Verlauf der Schlagfrequenz bei konstanten Temperaturen und bei Temperatursprüngen spricht sehr dafür, dass es sich um zwei antagonistische Prozesse handelt, die sich wechselseitig überwiegen. Der Verlauf dieser Prozesse wird auch durch Sauerstoffmangel beeinflusst, sowie auch durch Sympathicus- und Parasympathicuseinwirkung.

ZUSAMMENFASSUNG

Durch schnelle Temperatursprünge können in der Spontanrhythmik des Froschvorhofes überschüssige Tätigkeitserscheinungen ausgelöst werden, die besonders bei Ermüdung und Sauerstoffmangel zum Vorschein kommen. Die Befunde weisen darauf hin, dass es sich auch in den Schrittmacherfasern des Herzens um zweierlei Prozesse chemischer Art handelt, die antagonistisch sind und die sich wechselseitig überwiegen.

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AFFERENTE IMPULSE AUS DER ZUNGE BEI SÄURE- UND TEMPERATUREINWIRKUNG

von

KURT BOMAN

(Bei der Schriftleitung eingegangen am 27. October, 1959)

Die von Dodt und Zottermann (2) ausgeführten Untersuchungen über die Rezeptoren in der Zungenschleimhaut ergaben, dass in der Zunge Rezeptoren vorkommen, die bei Erwärmung der Zungenoberfläche eine gewisse phasische Zunahme der Spontanität aufweisen. Diese Rezeptoren werden von den Autoren als Warmrezeptoren bezeichnet. In den späteren elektrophysiologischen Versuchen konnten aber in den A-Fasern der Warmblüterhaut keine Rezeptoren dieser Art beobachtet werden, um so häufiger kamen aber besonders im Trigeminalggebiet der Katzen und der Ratten die sogenannten Kaltrezeptoren vor (1).

Da die Zungenoberfläche auch Geschmacksempfindungen übermittelt, und weil deswegen nicht mit Sicherheit eine gewisse Teilnahme der thermosensiblen Rezeptoren an Geschmacksempfindungen ausgeschlossen werden kann, wurde die Empfindlichkeit dieser Rezeptoren gegen Temperatur- und Säureeinwirkung gleichzeitig geprüft.

Diese Untersuchungen wurden an 10 Katzen durchgeführt. Narkose, Präparations- und Registrierungstechnik waren wie üblich (1). Bei den Versuchstieren wurde die rechte Seite der Mandibula rezesiert, Chorda tympani wurde vorsichtig aus dem Gewebe isoliert, abgetrennt und aufgesplittet. Auf die Zungenoberfläche wurde eine Wasserdurchströmte vergoldete Thermode aufgelegt. Die für die Säurereizung verwandte Essigsäure pH 2.6 wurde durch eine Kapillare in der Thermode auf die Zungenoberfläche gespritzt, dabei erhielt die Essigsäure genau dieselbe Temperatur wie die Thermode. Nach der Essigsäureeinwirkung wurde reines Wasser in grossen Mengen auf die Zungenoberfläche gespritzt.



Abb. 1. — Aktionspotentiale in einer dünnen Präparation der Chorda tympani der Katze bei Reizung der Zungenoberfläche. *a*, *b* Temperatursprünge, *b* Essigsäure (pH 2.5) einwirkung, durch einen Pfeil markiert, *d* Wärmesprung unter Essigsäureeinwirkung, *e* Temperatursprung nach der Essigsäureeinwirkung.

Die Abb. 1 zeigt das Verhalten der Rezeptoren der Zungenoberfläche bei Temperatur- und Säureeinwirkung. Ein Wärmesprung ruft in der Spontanitätigkeit dieser Fasern eine deutliche Zunahme der Tätigkeit hervor, wogegen ein Kältesprung gleicher Grösse und gleicher Temperaturänderungsgeschwindigkeit die Spontanitätigkeit hemmt. In der Abb. 1 *c* wird Essigsäure pH 2.6 auf die Zungenoberfläche langsam gespritzt, wobei eine geringe Impulszunahme beobachtet werden kann; unter andauernder Säureeinwirkung verschwindet die Impulszunahme aber bald. In der Abb. 1 *d* wird ein Wärmesprung unter der Einwirkung der Essigsäure gemacht, wobei deutlich beobachtet werden kann, dass keine Zunahme in der Impulstätigkeit mehr dadurch ausgelöst werden kann, ein Kältesprung bleibt gleichfalls wirkungslos. In der Abb. 1 *e* wird wieder ein Wärmesprung gemacht, nachdem die Zungenoberfläche mit reinem Wasser 5 min gespült wurde. Man sieht, dass die ursprüngliche Empfindlichkeit dieser Rezeptoren gegen die Temperaturreize wieder hergestellt ist.

Es wurde von uns beobachtet dass die Rezeptoren, die nach Art

eines Warmreceptors reagieren, unempfindlich gegenüber den Säure-
reizen bis pH 1.8 waren, sie zeigten also dieselbe Zunahme bei
Wärmereizen ohne und unter Säureeinwirkung. Stärkere Säuren
wurden von uns nicht verwandt. Die Säureeinwirkung rief meistens
in der Spontanitätigkeit der säure- und temperaturempfindlichen
Zungenreceptoren eine kurze, geringe Erregung hervor, die meistens
phasischen Charakters war. Eine Abnahme der Tätigkeit durch
Säureeinwirkung wurde nicht beobachtet.

Die Untersuchungen mit Kochsalz-, Zucker- und Chininlösungen
blieben erfolglos. Auf die Tätigkeit der Kaltreceptoren hatte die
Säure keinerlei Einwirkung. Die Warmreceptoren konnten durch
mechanische Reize nicht beeinflusst werden.

Nach diesen Untersuchungen kommen in der Zungenoberfläche
thermisch empfindliche Receptoren vor, die eine verhältnismässig
grosse Spezifität gegen die Wärmereize zeigen und können des-
wegen als Warmreceptoren bezeichnet werden. In wiefern sie aber
auf stärkere Geschmacksreize reagieren, blieb bis jetzt unbeant-
wortet. Wahrscheinlich handelt es sich hierbei auch um Receptoren,
deren Spezifität variabel ist, und die untereinander eine fliessende
Reihe von verschiedenen Receptoren bilden.

ZUSAMMENFASSUNG

In der Zungenoberfläche wurden Receptoren beobachtet, die
bei einem Wärmesprung eine phasische Zunahme aufwiesen, die
aber unter Essigsäureeinwirkung thermisch unempfindlich wurden.
Es wird vermutet, dass die Thermoreceptoren dieser Art auch von
variabler Spezifität gegenüber äusseren Reizen im physiologischen
Bereich sind.

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DAS VERHALTEN DER TRIGEMINUSRECEPTOREN BEI TEMPERATURREIZEN

von

KURT BOMAN

(Bei der Schriftleitung eingegangen am 27. October, 1959)

Die elektrophysiologischen Untersuchungen an Hautreceptoren im Trigeminusgebiet bei Hunden, Katzen und Ratten ergaben, dass in den thermosensiblen Hautreceptoren bei Erwärmung immer nur eine Hemmung der Spontanaktivität durch den Temperatursprung ausgelöst werden konnte, wogegen durch Abkühlung der Haut stets eine Erregung der Impulstätigkeit hervorgerufen werden konnte (1). In den an wachen Tieren durchgeführten Verhaltensversuchen (2) konnte trotzdem eine verhältnismässig grosse Wärmeempfindlichkeit der Gesichtshaut beobachtet werden. Das scheinbare Fehlen der sogenannten Wärmereceptoren veranlasste uns deswegen dazu, die einzelnen Nervenstämme im N. infraorbitalis systematisch durchzuuntersuchen, während auf den entsprechenden Gesichtshautgebieten Temperaturreize von ungefähr derselben Grösse und Form erzeugt wurden.

Diese Untersuchungen wurden an 11 Katzen und 4 Ratten durchgeführt. Präparation- und Registrierungstechnik waren wie üblich (1). Der ganze N. infraorbitalis wurde in der Orbita in dünne Bündel aufgesplittert, die nacheinander auf die Elektrode gelegt wurden. Die Temperaturreize wurden durch eine 75 W Heizspirale erzeugt, deren Abstand von der Hautoberfläche beliebig variiert werden konnte.

Abb. 1 zeigt einige Beispiele von den Registrierungen dieser Art. Bei jedem Versuch wurden 30 bis 50 Registrierungen gemacht. Abb. 1a und 1b zeigen einige Bündel mit 5—6 spontanaktiven

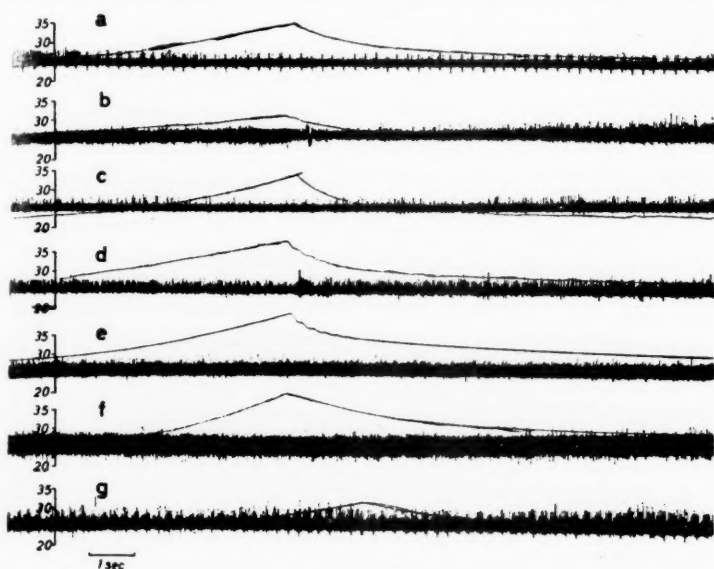


Abb. 1. — Aktionspotentiale in dünnen Präparationen des N.infraorbitalis der Katze bei Temperaturreizen. *a, b* thermisch empfindlich Fazern. *c, d, e* thermische weniger empfindliche Fazern. *f, g* thermische unempfindliche Fazern.

Fasern, deren Tätigkeit durch Erwärmung völlig gehemmt wurde, die der Erwärmung folgende Abkühlung ruft wieder die Tätigkeit hervor.

In den Abb. 1c, 1d und 1e sind dagegen Aktionspotentiale aus Nervenfasern zu erkennen, die den Temperaturreizen gegenüber unempfindlicher sind, allerdings kann auch hier eine gewisse Abnahme der Tätigkeit beobachtet werden.

Die Abb. 1f und 1g stellen das Verhalten der thermisch unempfindlichen spontantätigen Hautreceptoren bei Temperatursprüngen dar. Die völlige Unempfindlichkeit dieser Receptoren gegen Temperaturreize ist deutlich ersichtlich. Diese Fasern gehören zu der Gruppe der spontantätigen mechanosensiblen Hautreceptoren, die nur bei sehr hohen Hautoberflächentemperaturen (50—65°C) thermisch zu beeinflussen sind, bei diesen Temperaturen werden sie aber dann schon meistens irreversibel zerstört (3)

Es wurde von uns beobachtet, dass die Anzahl der temperatur-empfindlichen Receptoren bei der Ratte grösser ist als bei der Katze.

In diesen systematischen Untersuchungen wurde von uns nie-

mals beobachtet, dass durch Erwärmung der Hautoberfläche jemals eine Aktivitätszunahme in den Rezeptoren ausgelöst wurde. Durch die Präparation werden selbstverständlich viele Nervenfasern zerstört, aber es kann trotzdem nicht angenommen werden, dass ausschliesslich immer die Wärmerezeptoren zerstört würden.

Nach der ursprünglichen Hypothese von v. Frey wird u.a. vermutet, dass in den thermosensiblen Haut- und Schleimhautgebieten Rezeptoren vorkommen, von denen die Kaltrezeptoren Kälte, die Warmrezeptoren Wärme übermitteln. Zwar ist es für viele Forscher erstaunlich gewesen, dass die Empfindungsqualitäten Wärme und Kälte, die nur als relative Grössen physikalisch zu betrachten sind, durch ihre spezifischen Rezeptoren ins Bewusstsein übermittelt werden.

Da sämtliche in diesen systematischen Untersuchungen beobachteten Rezeptoren Kaltrezeptoren waren und weil ein Verhalten nach Art eines Warmreceptors nicht beobachtet wurde, wird von uns deswegen vermutet, dass die Temperaturnerven ein einheitliches Nervensystem bilden. Eine Wärmeempfindung wird ausschliesslich nur durch die Abnahme der Tätigkeit der spontantätigen Fasern ausgelöst, wogegen eine Kälteempfindung durch die Zunahme derselben hervorgerufen wird. Die von Boman und Hensel (4) durchgeführten Untersuchungen am Menschen ergaben auch bisher ein Fehlen der Warmrezeptoren.

ZUSAMMENFASSUNG

In systematischen Untersuchungen an Thermorezeptoren der Gesichtshaut konnte ein Verhalten nach Art eines Warmreceptors nicht beobachtet werden. Die Ergebnisse dieser Untersuchungen unterstützen die Annahme, dass es ein einheitliches Temperaturnervensystem gibt.

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INCIDENCE OF THE Gm SERUM GROUPS IN COLLAGEN DISEASES

by

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(Received for publication September 23, 1959)

After it had been demonstrated that a factor present in sera of some rheumatoid arthritic patients (RA factor) causes an agglutination of Rh-positive red cells coated with selected incomplete anti-Rh sera (2, 10), Grubb and Laurell made the observation that this reaction is inhibited by a part of normal human sera (4). The inhibitory factor was shown to belong to the gammaglobulin fraction and to be a stabile, dominantly inherited character. The phenotype which inhibits the agglutination is called Gm(a+) and the non-inhibitory phenotype is called Gm(a—).

It is apparently difficult to determine the Gm group of a rheumatoid arthritic patient, especially if the serum contains demonstrable amounts of RA factor at the same time. Grubb and Laurell have for this purpose found out a method of destroying RA factor by keeping the sera 10 minutes at 63°C. After this treatment the inhibitory substance of gammaglobulin still functions. The sera of rheumatoid arthritic patients have shown the same distribution of Gm groups as the normal population (3, 8). The total number of rheumatoid arthritic sera thus tested is 424. The study of the present material had begun before the publication of these two latter investigations. As the results did not quite agree with them, the work was continued.

¹ Aided by a grant from Finnish Rheumatism Foundation.

MATERIAL AND METHODS

The total number of patient sera tested was 473. 446 samples were from patients suffering from definite rheumatoid arthritis and 20 samples from other collagen diseases. The diagnosis was made in the Rheumatism Foundation Hospital, Heinola (398 cases) or in the Kivelä Hospital, Helsinki (68 cases).

The technique used is the same as was used for determination of Gm gene frequencies in the normal Finnish population (7).

The patient sera in the present work were blindly tested together with this earlier normal material, and several samples from many patients were also tested. All the sera showing the existence of RA factor were tested both untreated and after inactivation of 10 minutes in $+63^{\circ}\text{C}$. For standardization some sera of both Gm phenotypes, and one giving a weak Gm(a+), and some patient sera without RA factor, were simultaneously inactivated and tested.

RESULTS

There were 7 patient sera (1.5% of the material) of which the Gm group could not be defined, and when counting the frequencies they are not included.

The frequency of phenotype Gm (a+) in the total of 466 patients was 74.46% (347 cases) and that of Gm (a—) was 25.54% (119 cases). As the frequencies in the normal Finnish population tested simultaneously were 64.99% and 35.01% in the material of 477 unrelated persons (7) the difference is statistically significant.

Because the material of 20 different collagen diseases showed similar distribution of Gm groups as the rheumatoid arthritic sera they are not treated apart.

In the material coming from the Rheumatism Foundation Hospital there seems to be a greater deviation from normal frequencies than in the material coming from the Kivelä Hospital. The former showed Gm (a+) in 75.13 per cents, the latter in 70.29 per cents.

DISCUSSION

As Grubb says, Gm grouping of rheumatoid arthritic patients is not without difficulty (3). Partly this depends on the existence

TABLE 1

Gm GROUPS OF RHEMATOID ARTHRITIC PATIENTS AND HEALTHY ADULTS

	Gm(a +)		Gm(a —)		Total No. of Cases
	No. of Cases	Per-centage	No. of Cases	Per-centage	
RA factor not demonstrable with human O Rh + cells	323	76.36	100	23.64	423
RA factor demonstrable	24	55.81	19	44.19	43
Total of patients	347	74.46	119	25.54	466
Healthy adults	310	64.99	167	35.01	477

of RA factor, which must be inactivated in $+63^{\circ}\text{C}$. The circumstances in a serum after that procedure are not quite identical with those in a «native» serum. Some constituents taking part in the inhibitory reaction may have altered.

An alteration of Gm group happened in 3 rheumatoid arthritic sera where no RA factor could be shown. In two cases the «native» phenotype Gm(a—) altered to Gm(a+), and in one case an alteration from Gm(a+) to Gm(a—) took place.* When the sera were inactivated in $+63^{\circ}\text{C}$ for 10 minutes their reaction changed though it had been invariable and clear in many uninactivated samples from the same patient. When counting frequencies these cases could not be included. At any rate there seems to be a hint of some additional inhibitor, the possibility which is mentioned by Grubb, too (3). Variations in thermoresistance cannot be the only explanation for differences in Gm phenotype frequencies of normal and rheumatoid sera, because the difference was best visible in unheated sera where no RA factor could be demonstrated (Table 1). The Gm group of 43 sera containing RA factor must have been determined by inactivation method. The excess of Gm(a—) in them (19 cases, 44.2 per cents) agrees with the experience of Grubb (3). The numbers do not speak against a hypothesis of alternative inhibitors.

* In routine Gm determinations three additional alterations from Gm(a —) to Gm(a +) have been visible. Of these cases one was a normal serum and two were from patients suffering from febris rheumatica.

The use of normal sera as standards brings difficulty in Gm grouping of rheumatoid arthritic sera, too. The Gm group is a quantitative character determined by a dilution of the serum to be tested which still shows an inhibitory activity, and also in the normal material the power of this character slides individually. There is a possibility that the extra inhibition present in the sera of rheumatoid patients would be of some peculiar character. The presence of special types of inhibitors or a lack of them has been shown by Rantz *et al.* when comparing the normal and rheumatoid sera in Latex fixation test (9). Gray has demonstrated that in Waaler-Rose test the effect of RA factor on sensitized sheep cells can be neutralized by a thermolabile inhibitory substance which is present in normal sera and tissue extracts, but not in free form in rheumatoid arthritic sera (1). Still, the parallelity between Gm grouping and these other tests is not close, because Gm grouping depends on reactions of human constituents only without any need of extraneous particles.

The sera used as reactants, both the anti-Rh sera and rheumatoid sera used, may also vary individually. Grubb has the experience that different anti-Rh sera may cause variability in the results (3). Harboe and Lundevall have shown that different rheumatoid arthritic sera can detect two allelic genes behind the phenotype Gm(a+) (5). Differences in reactants may have led to different conclusions of Grubb's material and the present investigation.

During this work both reactants were invariable. They were the same as in studies of normal population. It seems that the only variable thing must have been in the material itself.

Any racial difference can hardly be pointed out in patients and normal individuals tested. In both of these groups there are persons originating from all parts of Finland.

A clinical study of the patients including serum electrophoresis may cast light to the problem and is under work. Whether there are genetically »non-specific» Gm(a+) reactions in rheumatoid arthritic patients would be seen after large studies concerning the Gm groups of their families. Also the existence of some genetical differences would be revealed only after Gm grouping the families of the patients, which Grubb regards as an important task, too (3). In these studies new types of rheumatoid sera distinguished by Harboe and Lundevall ought to be used (5, 6).

Rheumatology tells us that the disease very often tends to be inherited. Still, the present findings of difference between Gm phenotypes in normal and rheumatoid material need not mean any reflection of genetical difference.

SUMMARY

In the total of 466 patients suffering from collagen diseases the frequencies of Gm phenotypes show a statistically significant difference from the normal population. The etiology of this difference is discussed.

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5-HYDROXYINDOLE ACETIC ACID DURING PREGNANCY AND PUERPERIUM, IN NEWBORN INFANTS, AND GYNAECOLOGICAL TUMOURS

by

H. SAURAMO, P. REISELL, and V. BECKER

(Received for publication October 17, 1959)

In the course of the past decade it was observed that a malignant carcinoid of the small intestine or argentaffinoma can be diagnosed by chemical means. These tumours elaborate an indole derivative, serotonin (5-hydroxytryptamine, enteramine, serum vasoconstrictor) (2). It is transformed by oxidation deamination into 5-hydroxyindole acetic acid (5-HIAA). It is increased in the urine, and it ranges from 200 to 1000 mg. per day exceeding vastly the 5—10 (20) mg. per twenty-four hours in normal subjects.

A review of serotonin has been published recently (1), and so it does not seem necessary to deal with the literature in greater detail. Reserpine causes a discharge of serotonin. Chlorpromazine acts by blocking the sites at which serotonin is active. Bananas contain serotonin.

The urinary excretion of 5-HIAA shows individual variations. The excretion increases during pregnancy (6), decreasing again soon after delivery. The values are within normal limits. Serotonin does not seem to be related to delivery (3). In the case of premature separation of the placenta, serotonin may be of indirect importance (5). The excretion of 5-HIAA is normal at the menopause (4).

Granted by Sigrid Jusélius Stiftelse.

MATERIAL AND METHOD

Udenfriend's (7, 8) method was used in the present study, the 5-HIAA values in the urine being expressed as mg. per day. The test was made on 50 cases, once or several times. The diet did not include bananas. Determinations were made during pregnancy, in the puerperium, on newborn infants, and in cases of gynaecological tumours.

RESULTS

There is an increase in the values during a normal pregnancy. In the first trimester they are below 10 mg. daily. The same applies to cases of hyperemesis. During the second trimester the values range from 10 to 15 mg. daily, and in general this range is also obtained during the third trimester, though with larger individual variations. In one case of twin pregnancy the excretion was 25 mg. No differences were observed in connection with delivery. The values were similar in cases of toxæmia of pregnancy and hypertension, but an occasional value could exceed 20 mg. per day. In a case of eclampsia the rate of excretion was at the ordinary level. This was also the case in Rh-immunization. In the puerperium the value dropped below 10 mg. within one week. In one case in which reserpine was used during pregnancy the excretion was 40 mg. per day.

Newborn infants usually had an excretion of 0.2–0.6 mg. per day. During the first week there were individual variations, and the values also varied from one day to another in the same subject. There was no distinct sex difference.

In the case of gynaecological tumours the values ranged from 5 to 10 mg. per day, as a rule. The tumours were: uterine myoma, ovarian fibroma, dysgerminoma, thecoma, malignant teratoma and adenocarcinoma. The rate was between 10 and 15 mg. in only one case of ovarian adenocarcinoma and in 2 cases of generalized carcinoma of the abdominal cavity.

DISCUSSION

During pregnancy the 5-HIAA excretion in the urine increases to some extent, which must be regarded as physiological. Delivery

does not affect the excretion, and in the puerperium the values soon return to the non-pregnancy level. In some pathological cases there are no essential differences.

Calculated per kilogram of body weight, the values for the newborn are the same as for adults. Expressed in terms of milligrams, adults show a value 20 times as high.

As far as the gynaecological tumours are concerned, determinations of 5-HIAA seem to lack clinical importance.

In general it would seem that serotonin and 5-HIAA have no direct clinical importance in gynaecology and obstetrics. Serotonin seems to be a hormone specific for the enterochromaffinic system

SUMMARY

The urinary excretion of 5-hydroxyindole acetic acid, transformed from serotonin by oxidation deamination, shows an obviously physiological increase during pregnancy and returns to the non-pregnancy level soon after delivery. Expressed per kilogram of body weight, the excretion in newborn infants corresponds to the values for adults. In cases of gynaecological tumours the excretion is within normal limits.

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THE NATURAL CLEANING OF THE TEETH SURFACES

by

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(Received for publication October 15, 1959)

It is well known fact that the caries frequency varies considerably according to the various teeth surfaces. The lingual surfaces show distinctly less caries than the buccal surfaces (4, 7). In the discussion of the obviously complex pathogenesis of caries remarkable attention has been paid to the correlation between the lactobacillus count and the caries disease (3, 6, 8), although such correlation could not (9) always be established. It seems (2) that in a carious area the dental plaques contain more lactobacilli than on healthy dental surfaces. By means of staining the teeth with fuchsin, Arnim (1) has shown in a perspicuous manner the loci of retention of microorganisms.

The purpose of the present work was to throw some light upon how the teeth are cleaned in natural conditions.

MATERIAL AND METHODS

The cleaning of the teeth was studied by means of staining the teeth with a solution of Chinese ink. Ten candidates in Dentistry (8 women and 2 men) volunteered in the experiment. Their teeth was inspected, dried with air and covered by brushing with a uniform coat of Chinese ink. After about $1\frac{1}{2}$ to 2 hours the teeth were re-inspected and the teeth surfaces that had become cleaned were recorded. No meals were taken during the intervening time.

The bacterial sample was taken with a sickle-shaped remover from the buccal and lingual surfaces of the lower molars, making one stroke over the buccal and lingual surface, respectively, over its entire length. The sample was suspended in 1 ml of saline, the tube was agitated during 2 minutes and serial dilutions in powers of 2 or 10 were made. Each dilution was cultivated on a tomato juice dish (Difco), for 78 hours at 37°C. Subsequently the lactobacillus colonies were counted.

RESULTS

In all cases the cleaning of the teeth, which is primarily due to the action of musculature and saliva, showed a similar trend in that the lingual surfaces were cleaned considerably more quickly than the buccal surfaces. In most instances all lingual surfaces were completely clean after 1½ to 2 hours (Figs. 1 to 2). The teeth in abnormal positions were slowly cleaned. Some variations occurred on the labial side in the incisor region: The incisal part of the incisors on the labial side was usually cleaned first of all. In two instances the gingival part was cleaned first.

In all instances the relative number of lactobacillus in samples taken from the buccal surfaces of the lower jaw was higher than that from the lingual surfaces of the same teeth (Fig. 3). The difference was roughly ten-fold.

DISCUSSION

The self-cleaning of the lingual surfaces can be primarily referred to the cleaning action of the tongue, while the cleaning action of cheeks is not equally effective. It is conceivable that the different shape of the buccal and lingual surfaces also affects their cleaning. The rapid self-cleaning of the lingual surfaces and their lower lactobacillus count in comparison with the buccal surfaces and the fact that the lingual surface also shows a lower caries frequency would explain why retention areas are more common on the buccal side.

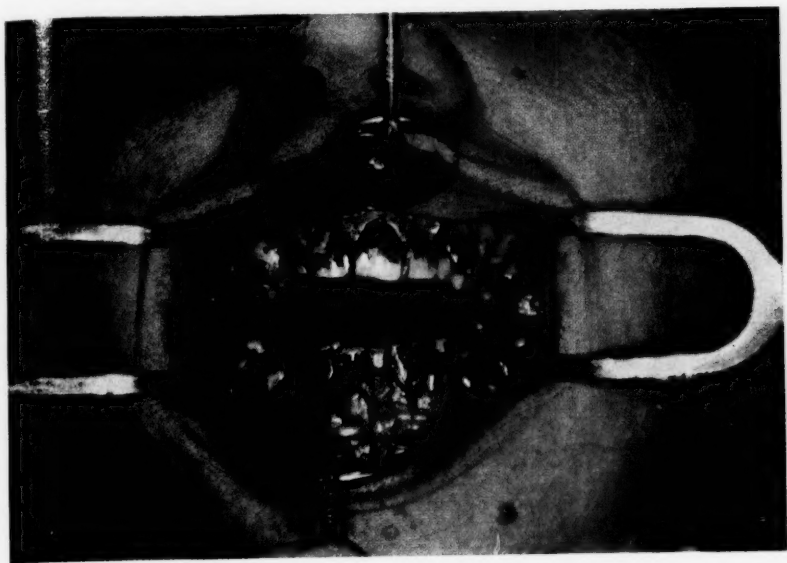


Fig. 1. — The teeth of a test subject, two hours after staining. The buccal surfaces are still black. The incisal part of the incisors has become cleaned on its labial surfaces.

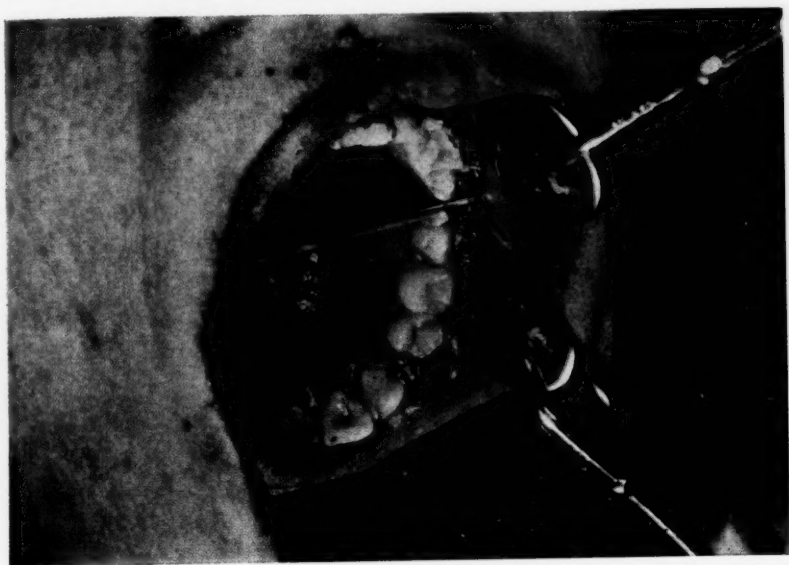


Fig. 2. — The teeth of the same subject. The picture is taken at the same time as the picture no 1. Palatal and occlusal surfaces have become cleaned. The canine tooth (3) in the labial position has remained uncleaned.

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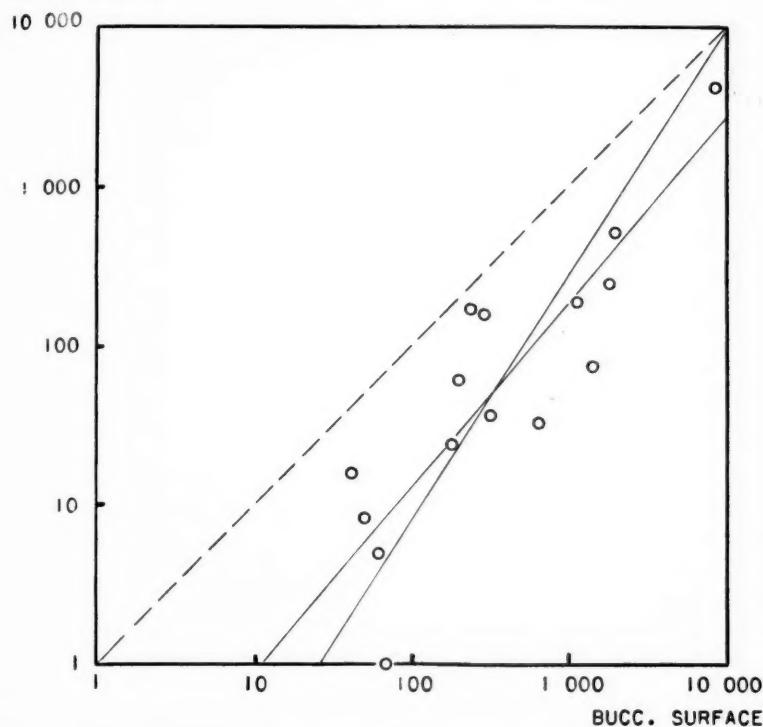


Fig. 3. — Relative lactobacillus count of the buccal and lingual surfaces of the same molars with 15 subjects. (Logarithmic scale for both bacillus counts).

Coefficient of linear correlation between the logarithms of lingual and buccal count: 0.87.

Solid lines: Lines of regression.

Dotted line: Equal count on buccal and lingual surfaces.

SUMMARY

The natural cleaning of the teeth has been studied (with ten subjects) by staining the entire tooth with Chinese ink and inspecting it after $1\frac{1}{2}$ to 2 hours without intervening meals. As a parallel study the lactobacillus counts on the buccal and lingual surfaces of the molar region of the lower jaw (with 15 subjects) were performed.

In all instances the lingual surfaces became cleaned more

quickly than the buccal surfaces. The teeth in abnormal positions were rather slowly cleaned. Variations were observed on the labial surfaces of the incisors.

The lactobacillus count was roughly 10 times higher on the buccal surfaces of the lower jaw than on the lingual surfaces of the same teeth.

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REPLACEMENT OF EXTRARENAL FLUID LOSS

by

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(Received for publication October 24, 1959)

A situation often arises in the treatment of surgical patients in which the extrarenal loss of fluid is such both quantitatively and qualitatively that it upsets the normal fluid balance. The situation is especially difficult when oral intake is prevented by the patient's disease. Disturbances of intestinal resorption, vomiting, intestinal sinuses and the use of gastrointestinal suction may rapidly result in disorders of fluid balance. The use of suction is justified in conditions in which intestinal decompression is necessary. Episodes of vomiting may also be avoided by suction, and this measure likewise assists the recovery of fluids of the alimentary canal for careful quantitative and qualitative replacement of losses. Determination of the quality of extrarenal fluid loss requires laboratory determinations which are often time-consuming and which not all hospitals can perform. Serviceable «bedside» methods have been introduced for chloride, but chloride determination alone is not sufficiently illustrative of the fluid loss (5). I have carried out examinations of extrarenal fluids on patients at the Surgical Department of the Regional Hospital of Central Finland, both for electrolyte content and for acidity. This is a report on the results obtained. It may clarify certain points to be considered in the replacement of extrarenal fluid loss.

MATERIAL AND METHODS

The determinations were performed on 36 patients, giving 65 samples:

nasogastric suction fluid	54 samples	from 31 patients
bile	4	» » 2 »
pancreatic juice	3	» » 1 patient
ascites	2	» » 2 patients
small intestinal juice	1 sample	» 1 patient
chyle	1	» 1 »

65 samples

One ascites and small intestinal juice determination was made on patients whose nasogastric suction sample was examined. Indications for the use of suction were occlusion (11 cases), peritonitis (9 cases), cancer of the alimentary canal (6 cases) and miscellaneous diseases (atresia of esophagus, pancreatitis, gastric ulcer, etc). The suction fluid was not merely gastric juice; it also included sputum and possibly regurgitated bile and intestinal juice.

The sodium and potassium concentrations of the fluids were determined by the EEL direct-reading flame photometer method, the chloride concentrations by the method of Schales and Schales (4), and the acidity by an electrical pH meter with a calomel electrode (Radiometer). In the treatment of the material the results were classified according to pH: one group comprised cases in which $\text{pH} \leq 3.5$, with free hydrochloric acid: a second was $\text{pH} = 3.6\text{--}6.9$, acid fluids; the third was $\text{pH} = 7.0$ and over, neutral and alkaline fluids. This classification applied only to fluids aspirated by nasogastric suction for which statistical methods could be used to some extent in analysing the results.

RESULTS

The table shows the electrolyte concentrations of the fluids obtained by *nasogastric suction*, distributed into pH groups. Only three samples had a pH of 7.0 and over and their electrolyte concentrations are mentioned separately.

TABLE
ELECTROLYTE CONCENTRATIONS OF FLUIDS OBTAINED BY NASOGASTRIC SUCTION
IN pH GROUPS ≤ 3.5 AND $\text{pH} = 3.6\text{--}6.9$

	$\text{pH} \leq 3.5$		$\text{pH} \leq 3.6\text{--}6.9$	
Sodium mEq/l	73.0	± 6.8	93.3	± 5.2
(range)	(36—130)		(18—138)	
Potassium mEq/l	8.4	± 0.92	6.8	± 0.73
(range)	(1.9—14.6)		(1.4—20.0)	
Chloride mEq/l	124.0	± 5.0	94.4	± 6.1
(range)	(75—151)		(24—129)	

The electrolyte concentrations in each pH group gave the anticipated sodium-chloride ratio — in more acid fluids the mean chloride concentration was very significantly greater than in less acid fluids. A concentration under chloride serum. Concentration 108 mEq/l, was found in only 3 of the 23 samples in the group $\text{pH} \leq 3.5$, in 16 of the 27 samples in the group $\text{pH} = 3.6-6.9$. In both groups the sodium concentrations were lower than the serum concentration, in the group $\text{pH} \leq 3.5$ nearly significantly lower than in the group $\text{pH} = 3.6-6.9$. No significant difference could be established in the potassium concentration of these groups. A concentration higher than 10 mEq/l (= 2 times the «normal» serum concentration) was found in the group $\text{pH} \leq 3.5$ in 10 out of the 23 samples, and in the group $\text{pH} \leq 3.6-6.9$ in 5 out of the 27 samples. This difference, too, was of no statistical significance. The potassium concentration was under 5 mEq/l in 13 of the 50 determinations (26 per cent). The potassium concentration of fluids obtained by gastric suction was thus in general higher than the «normal» serum concentration.

In the three cases of the group $\text{pH} = 7.0$ and over, the sodium concentrations were 82, 86 and 106 mEq/l, potassium concentrations 12.0, 3.0 and 4.8 mEq/l and chloride concentrations 64, 100 and 65 mEq/l, all of them values which do not differ essentially from the electrolyte concentrations of mildly acid gastric solutions.

The correlations between the electrolyte concentrations and the degree of acidity were also calculated. The correlation was $+0.50 \pm 0.10$ for sodium, -0.59 ± 0.09 for chloride and -0.28 ± 0.13 for potassium. In other words, when the pH rises the sodium concentration also increases and the chloride concentration decreases. As regards potassium, the negative correlation is statistically only almost significant.

The general procedure was to examine a maximum of two samples from one patient. In each case the acidity of the gastric juice kept within a given group. In one case, however, 9 determinations were made on different days. The graphic representation of the concentrations and pH variations for this patient is given below.

The figure shows how the sodium and chloride concentrations remained virtually unchanged for many days. An increase in the sodium and a decrease in the potassium concentration with the rise in pH was observed on the last day.

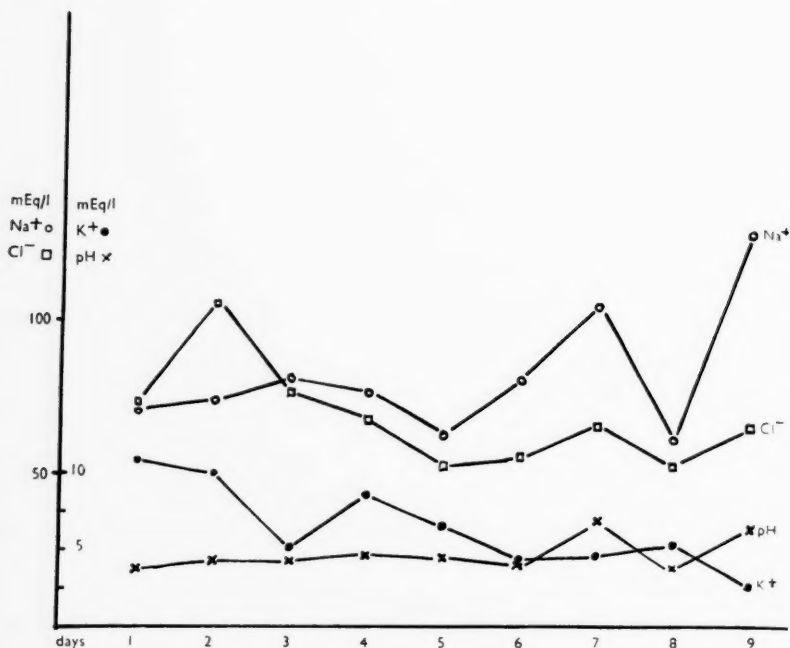


Fig. 1. — Sodium, potassium and chloride concentrations and pH values in samples taken by nasogastric suction from one patient.

The pH values of pancreatic fluid, small intestinal fluid and chyle were over 7.0 and over 6.0 in bile. In ascites it was 3.8. The electrolyte concentrations in these fluids ranged: sodium 60–190 mEq/l, potassium 2.7–10.8 mEq/l and chloride 69–126 mEq/l. In seven of ten determinations the concentrations were reminiscent of normal serum concentrations. The correlation of sodium concentration to pH value was highly significantly positive ($+0.72 \pm 0.17$). No correlation was found between potassium or chloride concentrations and pH value.

DISCUSSION

There are not many reports on the correlation between the electrolyte concentrations of gastrointestinal fluids and acidity. The differences found in the electrolyte contents of gastric fluids of gastric ulcer and gastric cancer patients are referable to differ-

ences found in more and less acid fluids. Insulin and histamine studies have shown that the potassium level in gastric fluid of hyposecretors is higher than that of normal controls (1, 3). No definite correlation supporting a corresponding finding was established in the present investigation in which the fluid examined was undifferentiated gastric fluid containing also, say, sputum. Nor did Lans *et al.* (2) establish any differences in the potassium concentration of the gastric juice of gastric ulcer and gastric cancer patients. Bernstein (1) found that the sodium and potassium of gastric juice generally decreased and chloride increased with a rise in acidity. According to my own investigations, this was established for sodium and chloride but not for potassium. Determination of the acidity of gastric juice concurrently with the chloride concentration gave a clear idea of the composition of the fluid loss in question. This is all the more important if we take into consideration the strain on the acid-base balance involved in the excretion of, for instance, acid gastric juice. Indicator papers suffice to determine the acid level and no large laboratory is necessary (5).

The small number of other fluids lost extrarenally which were studied here do not warrant any conclusions. However, normal serum concentrations may be used as a basis for assessment of the nature of the fluids and for replacement of those fluids by isotonic solutions. Determination of the degree of acidity gives considerable additional information regarding the replacement of those fluids.

Potassium which may have a high, on occasion even a very high, concentration in gastric juice is also always involved in extrarenal fluid loss. Numerous investigations have shown that potassium, at any rate intracellular potassium, is lost in operated patients, and hence special attention must be paid in post-operative cases to the replacement not only of sodium and chloride but also of potassium if there is extrarenal fluid loss by suction or by other routes.

SUMMARY

A study was made of fluids obtained principally by nasogastric suction (54 samples from 31 patients) from patients treated at the Surgical Department of the Regional Hospital of Central Finland.

Since there is a certain correlation between the sodium, potassium and chloride concentrations of these fluids and their acidity, pH determination was suggested as a simple method for use with chloride determination to assess the replacement required for extrarenal gastrointestinal fluid loss. This method makes it possible to administer the therapy required with a fair degree of accuracy and without the need for the more time-consuming determinations of sodium and potassium. It is suggested that the idea deserves consideration especially as these patients are often treated in hospitals with laboratories which lack the facilities for the determination of sodium and potassium.

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INFLUENCE OF INDOLACETIC ACID AND TISSUE HOMOGENATE ON THE MITOTIC ACTIVITY OF SOME ORGANS OF THE RAT¹

by

ANTTI ALHO

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In plants many hormone-like substances have been found, a group of which are called growth hormones. The group phytohormones known for the longest time is the auxins, of which 3-indolyl-acetic acid (IAA) is the most important. IAA has also been found in animal organisms. Kögl was the first to extract it from urine. 2,4-dichlorophenoxyacetic acid (2,4-D) also is an auxin. Another group is called kinines according to kinetin (6-furfuryl-aminopurine).

Phytohormones have in plants greatly divergent influences on cell division, growth and differentiation, sometimes stimulating, sometimes inhibitory, depending on the concentration of the effective substance and on the tissue (1).

The influence of growth hormones of plants as growth factors in animal cells has also been examined. Roul *et al.* (2) found that IAA is a vitamin-like food for the rat. Dunning *et al.* (3) showed that IAA, like tryptophan and indole, increases the incidence of 2-acetaminofluorene-induced carcinomata in rats, and Hartung (4) showed a stimulation of tumorous aggregations in *Drosophila* larvae. Louis (5) investigated the influence of IAA on tissue cultures of chicken embryo heart and found that low concentrations have, up to a certain limit, a stimulating effect and higher concen-

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trations an inhibitory effect on growth. Apffel (6), using large doses, examined the cytostatic effect of 2,4-D on some human tumours. Ogawa *et al.* (7) produced a mitotic stimulation in Yoshida sarcoma cells of the rat, and Guttman *et al.* (8) an acceleration of division in *Paramecium caudatum* using kinetin. Lettré *et al.* (9) found no stimulation by kinetin in the growth of cultures of normal and malignant human and animal cells.

As in the plant investigations (1), an optimum concentration was essential in many of the aforementioned investigations. This concentration causes the greatest stimulation, whereas large doses restrain the growth phenomena.

Using intraperitoneal injections of homogenate of orbital gland tissue of the rat, a stimulation of mitotic cell division was observed in homologous organs (10, 11). The importance of the amount used, some tenths of a milligram, was emphasised, for no stimulation is elicited with too small or too large amounts. A parallelism existing in the nature of the effect of different growth promoting substances on plant and animal tissues was found.

The purpose of the present investigation was to study whether IAA, or heteroauxin, is capable of increasing the mitotic division in some tissues of the rat, and how this ability is manifested in the presence of a tissue-specific stimulating agent.

MATERIALS AND METHODS

Experiment 1. — Forty 6 to 7 weeks old rats of Wistar strain, weighing on an average 75 gm, were used. The experimental solutions consisted of 0.18 mg of IAA (Indol-3-yl-acetic Acid, Horticultural Quality, BDH) in 1 ml of physiological saline (10^{-3} M solution) and of 0.5 mg of outer orbital gland tissue of 2 weeks old rat homogenised in 1 ml of saline solution. The IAA solution was prepared by dissolving crystalline IAA in a few drops of ethanol and adding saline. Intraperitoneal injections were made as follows: Control group, physiological saline; 2 groups, IAA; 2 groups, tissue homogenate; 2 groups, IAA and tissue homogenate. The rats were decapitated at 19–20 o'clock on the third or fourth days from the beginning of the experiment (table 1). In groups 1, 2 and 3, samples were taken from the skin and the glandular stomach, and in all the groups one of the outer orbital glands was

TABLE 1

INCIDENCE OF MITOSES PER 10,000 CELLS (MEAN \pm STANDARD ERROR) IN EXPERIMENT 1

Group No.	No. of Animals	Procedures	Mitotic Indexes		
			Orbital Gland	Epi-dermis	Glandular Stomach
1	10	Saline, 5 rats killed 3rd day, 5 rats killed 4th day	3.2 ± 0.8	17.5 ± 3.0	13.5 ± 2.4
2	5	IAA 1st and 2nd day, rats killed 3rd day	8.5 ± 2.2	11.3 ± 3.7	5.9 ± 1.4
3	5	IAA 1st, 2nd 3rd day, rats killed 4th day	2.4 ± 1.4	11.1 ± 2.0	14.4 ± 1.7
4	5	Homogenate 2nd day, rats killed 3rd day	6.3 ± 2.2		
5	5	Homogenate 2nd day, rats killed 4th day	3.6 ± 1.2		
6	5	IAA 1st and 2nd day, homogenate 2nd day, rats killed 3rd day	7.0 ± 2.4		
7	5	IAA 1st, 2nd and 3rd day, homogenate 2nd day, rats killed 4th day	6.0 ± 1.7		

removed. In groups 1 and 2, the distribution of mitotic phases was calculated.

Experiment 2. — This was a supplementary experiment to study the influence of IAA on the orbital gland. Eight rats of Wistar strain, 5 to 6 weeks old and weighing about 50 gm, were used. The same IAA dosage and time of decapitation as in Experiment 1 were employed (table 2).

The tissue samples were fixed in Bouin's solution and the 4 μ thick sections were stained with haemalum-eosin. The counting of

TABLE 2

INCIDENCE OF MITOSES PER 10,000 CELLS (MEAN \pm STANDARD ERROR) IN EXPERIMENT 2

Group No.	No. of Animals	Procedures	Mitotic Indexes
			Orbital Gland
8	3	Saline, rats killed 2nd day	1.5 ± 0.3
9	5	IAA 1st and 2nd day 10.00 a.m., rats killed 2nd day	4.9 ± 1.8

mitoses in the orbital gland was done by measuring first an area containing 500 cells and by then counting the mitotic figures in an area of 10,000 cells. The counts in the epidermis and the glandular stomach were done according to the same principle. To make the variations in the small test groups as small as possible, early pro-phases and late telophases were excluded from the count because of the errors possible in determining these stages.

In the orbital glands the parenchymal cells were counted, in the skin the nucleated epidermal cells, and in the glandular stomach the mucous neck cells.

RESULTS

Using Student's *t*-test the significance of the differences in the orbital gland between groups 2 and 3 and between groups 1 and 6+7 ($n = 10$, $m = 6.5$, $s.e. = 1.4$) and in the glandular stomach between groups 1 and 2 were found to be of the order of $P < 0.05$. Between the glandular stomach groups 2 and 3 the difference was at the level of $P < 0.01$.

When the two control groups and all the groups given IAA (2, 3 and 9) are combined, the significance of the difference between the two sets of groups is $P < 0.05$. Calculation of the distribution of the various mitotic phases in the orbital gland groups 1 and 2 revealed no significant differences.

CONCLUSIONS AND DISCUSSION

It is observed that IAA has a stimulating effect on mitotic division in the orbital gland. The finding of a similar distribution of different mitotic phases in the control and experimental groups indicates that this is not a colchicine-like effect.

In the glandular stomach, cell division was inhibited by IAA. The reversion of the mitotic frequency in group 3 to the control level in the orbital gland and the glandular stomach cannot be explained on the basis of the available material.

No effect was observed in the epidermis.

When IAA and tissue homogenate were administered simultaneously, the mitotic stimulation was more clearly pronounced and of longer duration than when either of these substances was used alone.

The present work corroborates the previous observations that plant growth factors influence also animal cells. The difference seen in the effect on cells of the orbital gland and on cells of the glandular stomach may be due to a difference in the sensitivity of these tissues to the dose of IAA used, which was of the same order as that employed by Louis (5).

SUMMARY

The stimulating effect of indolacetic acid (IAA) on mitotic activity was studied in the outer orbital gland, the epidermis and the mucous neck cells of the glandular stomach of the rat. The combined effect on the orbital gland of IAA and orbital gland homogenate of young rats was also investigated.

IAA in the dosage used stimulated mitotic activity in the orbital gland and inhibited it in the glandular stomach. No effect was seen in the epidermis. The combined effect of IAA and organ homogenate was also stimulative.

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FLAVASPIDIC ACID GLUCAMINATE

ITS INHIBITORY EFFECT ON THE ADENOSINTRIPHOSPHATASE ACTIVITY
OF THE CARDIAC MUSCLE OF RAT STUDIED IN VITRO

by

TOIVO MARKKANEN, MARTTI PULKKINEN, and HEIKKI SALMI

(Received for publication November 13, 1959)

It is known that male fern substances have a particularly poisonous effect on the protoplasm of contractile cells (11). The characteristics of the mentioned substances have been known to affect electrocardiographically the changes in the cardiac muscle which various research workers have observed in connection with clinical tapeworm eviction (4, 6). Examinations of isolated cardiac muscle in laboratory tests have yielded negative inotropic and chronotropic effects (7, 8). Intravascular administration of the substances mentioned have caused a pressure rise in the right ventricle, as is the rule, and the consequent muscular injuries have brought about electrocardiographic changes (9). Endeavouring to clarify more closely the pathological mechanism of the mentioned changes in the cardiac muscle in the present investigation, we have studied the effect of N-Methylglucamate of flavaspidic acid on the adenosintriphosphatase¹ activity on cardiac muscle of rat in vitro.

METHOD

Calcium activated adenosintriphosphatase was determined according to Perry (12). For the tests, the cardiac muscle of 7 Wistar

* The writers are indebted to Pharmaceutical Manufacturers «Leiras» for the financial aid.

albino rats were used; Directly after killing the rat the muscle homogenates were prepared in a glass homogenizator at $+4^{\circ}\text{C}$ with 0.5 molar potassium chloride. To the solution containing of 0.5 ml 0.2 molar glycine buffer pH 9.1, 0.1 ml, of 0.1 molar CaCl_2 and 0.15 ml of 0.05 molar adenosintri-phosphate (as dinatrium salt and neutralized by 0.2 normal sodium hydroxide to pH 6.8) was added 0.15 ml of a water solution of N-Methylglucamine of flavaspidic acid the different concentrations to be studied. The solution thus obtained was pre-incubated at 25°C . for 3 minutes after which 0.1 ml of 2 per cent of the mentioned cardiac muscle homogenate was added. After incubation at 25°C . for 10 minutes the reaction was stopped by 0.5 ml of 15 per cent trichloroacetic acid. The precipitate was centrifuged, and from the clear filtrate the liberated phosphate was determined by Fiske-Subbarow's method (3). Further, by means of the same method, determinations of the phosphate content were separately made of the cardiac muscle homogenate, reagent and flavaspidic acid glucamine. One double determination of each homogenate was made in each test series, in which there had been no flavaspidic acid glucamine what so ever. For the final concentration of flavaspidic acid glucamine, 1, 5, 10, 20 and 200 mg per cent was used. The effect of the corresponding glucamine solution on the adenosintri-phosphatase activity was also made by means of the method described. Double determinations were always made. —

RESULTS

In Table 1, the results of the tests are given as follows: the concentration of each flavaspidic acid glucamine corresponds to the amount of liberated phosphate in μg , which reveals the relative adenosintri-phosphatase activity prevailing in these test conditions during a fixed period. The glucamine together with which flavaspidic acid forms, combined in the water solution, was found not to have any effect at all on the activity of the adenosintri-phosphatase. The phosphorus content of the cardiac muscle homogenate was $0.6 \mu\text{g}$, on an average; determinations were required at different points. From the results it is seen that the reduction of the adenosintri-phosphatase activity in the diluted flavaspidic acid glucamine solution, here in question, was very small, and

TABLE 1

THE FLAVASPIDIC ACID GLUCAMINATE CONCENTRATION AND CORRESPONDENT
LIBERATED PHOSPHATE IN $\mu\text{g}/10$ MIN.

N-Methylglucamine of Flavaspidic acid in mg %	Liberated Phosphate in $\mu\text{g}/10$ Minutes						
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Aver- age
0	30.40	29.49	24.00	7.52	28.29	27.09	24.46
1	30.70	36.42	24.68	4.51	22.57	21.97	23.47
5	30.10	26.48	17.75	4.21	20.76	17.45	19.46
10	21.97	15.05	11.73	1.50	15.65	11.43	12.89
20	12.97	6.62	8.12	1.50	8.72	8.12	7.67
100	4.81	1.20	0.60	0.00	1.20	1.50	1.55

only on an average of a 4 per cent magnitude, calculated from the value when none of the mentioned substances were used in the tests. When the concentration of the substances studied was five times stronger in the solution, the inhibition value of the enzyme was also about fivefold. On increasing the amount of inhibitory substance by 10, the inhibition percentage of adenosintriphosphate rose in about the same proportion. The same occurrence was observed if the flavaspidic acid glucamine reaction volume was increased by 20; in these test conditions the greater part of the enzymes to be dissolved the adenosintriphosphate had been inhibited. A 100 mg per cent solution caused practically total inhibition. When a 1—20 mg per cent flavaspidic acid glucamine solution was used there was thus a linear, increased growth in the inhibitory effect on adenosintriphosphatase.

DISCUSSION

Previous investigations show that male fern substances have an injurious effect on the activity of the cardiac muscle: the contraction of the isolated cardiac muscle have become weaker and the spontaneous rhythm is slower. The effect of flavaspidic acid generally appeared on relaxation of the muscle preparation and spontaneous contraction was more difficult. In our efforts to clarify the mechanical action of the cardiac muscle in the tests it was observed that the weakest flavaspidic acid concentration which still distinctly weakened the spontaneous contraction was 0.17 mg per cent (9).

It is known that the dissolution of adenosintriphosphate and the enzymes which cause the dissolution have their central point in muscle contraction. One of these enzymes is Ca-activated ATP-ase (myosin ATP-ase). However, its activity determinations in raw homogenates interfere to some extent with other phosphatases, arypases and with the acid labile phosphates of the mogogenates (2). Study of pre-incubation of the enzymes and inhibitor are of importance from the point of view of acceleration or slowing down of the reaction (12). Consequently, on study of the adenosintriphosphatase activity, only the relative value and the serial results obtained by the same method are mutually comparable. In each series, the method here used has yielded results pointing in the same direction and may thus be considered reliable.

A possibility that the flavaspidic acid weakens the activity of the cardiac muscle (negative inotropic and chronotropic action) is evidently the fact that the substance mentioned causes inhibition of the adenosintriphosphatase activity.

The flavaspidic glucamate concentration necessary for inhibition is comparable with other inhibitory substances, and only small quantities are required (10). The lowest flavaspidic and glucamate content used in this investigation to cause inhibition was one mg per cent. Whether the observation made in this study is of significance with regard to the cardiac complications noted in connection with clinical tapeworm eviction cannot be definitely established. We know, however, that anoxemia has a weakening effect on the adenosintriphosphate dissolution (1). If the patient who has previously had nutrition difficulties of the cardiac muscle is given tapeworm medicines, detrimental to the adenosintriphosphatase activity, the result may be inhibition of the biochemical activity of the cardiac muscle. Nothing is definitely known, however, about absorption of the Filix-substances but it evidently does occur, as cases of poisoning have been reported after intake of these substances. In connection with clinical tapeworm eviction, it may happen that such quantities of the Filix-substances are absorbed from the intestine into the blood circulation that, for instance, inhibition of the adenosintriphosphatase activity in the cardiac muscle occurs to some extent. What role the detoxication system of the organism plays as a destroyer of the Filix-substances is not known.

SUMMARY

The inhibitory effect of N-Methylglucamine of flavaspidic acid on the activity of adenosintriphosphatase has been studied in vitro. The substance was found to have a comparatively strong inhibitory effect on the adenosintriphosphatase activity.

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EFFECT OF PANTOTHENIC ACID ON THE ACETYLCHOLINE SENSITIVITY OF THE ISOLATED RAT INTESTINE

by

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The significance of pantothenic acid, a member of the vitamin B complex, in vitaminology has been stressed by many workers during the past decade (4, 8, 12, 23, 28, 30, 33, 36, 37, 41, 42, 44, 48, 52). Although many aspects of its action, physiological and pathophysiological, are still unclear, it is the common view that its most important function is the participation in acetylation processes (15, 22, 44, 55); as a component of coenzyme A it is involved in the conversion of choline to acetylcholine. As it is known, acetylcholine is the most important mediator of myoneural transmission and as such is one of the most important stimulants of intestinal motility. In addition, pantothenic acid takes part in the catabolism of carbohydrates, in protein synthesis, in the anabolism and catabolism of fats, and, which is also remarkable, in the formation of steroid hormones. Its epitheliumprotecting action has been also emphasized.

The role played by pantothenic acid in ensuring intestinal motility is indicated among others by the intestinal changes observed in experimental pantothenic acid deficiency: intestinal peristalsis, tone and responsiveness to drugs are diminished (3, 24), the intestinal mucosa exhibits severe histological changes (46, 54). Recently, emphasis has been laid on the symptoms of adrenal

cortical hypofunction, that occur among the other deficiency symptoms (2, 13, 17, 26), with the disturbance in the balance of electrolytes important in maintaining normal peristalsis. The above mentioned changes disappear with the disappearance of pantothenic acid deficiency.

On the basis of these observations pantothenic acid has been successfully employed in the treatment (1, 9, 10, 11, 14, 18, 20, 22, 25, 34, 35, 38, 45, 51) and prevention (9, 24, 27, 39, 40, 43, 45) of paralytic ileus, although the experimental pantothenic acid deficiency causes no major changes of intestinal activity in man (19, 50). We have found the use of pantothenic acid advantageous in the treatment of intestinal paralysis of infants and children (49).

As the mechanism of the action of pantothenic acid on gastrointestinal tone and motility is a rather controversial issue, and in view of the important mediator action of acetylcholine on the maintenance and initiation of peristalsis, it has been deemed interesting also from the point of view of practice to study the action of pantothenic acid on the acetylcholine-sensitivity of the intestine.

METHODS

24 normal Wistar rats fed a mixed diet and averaging 200 g in weight were used. Most of the experiments were made during the spring months. The animals were killed by a blow to the occiput, the specimens of small intestine were suspended in a Magnus vessel containing 12 ml of Tyrode's solution, through which air was flown by means of a membrane pump. The water bath around the Magnus vessel had a temperature of $38 \pm 0.2^\circ\text{C}$. The longitudinal movements of the intestine were recorded kymographically. The test substances were dissolved in Tyrode's solution, adjusting the pH to 6.9 — 7.2 by adding a few drops of $n/10$ HCl or sodium hydroxide. The pH was controlled by means of an universal indicator. To eliminate differences due to fluid pressure, the volume of the Tyrode's solution was constant. Error due to individual sensitivity and differences in the acetylcholine content of the various segments of the rat intestine (5) was counteracted by determining in every experiment the acetylcholine concentration producing the same deflection (25 mm) on the kymograph, when applied repeatedly to the same specimen of intestine. This concentration varied from

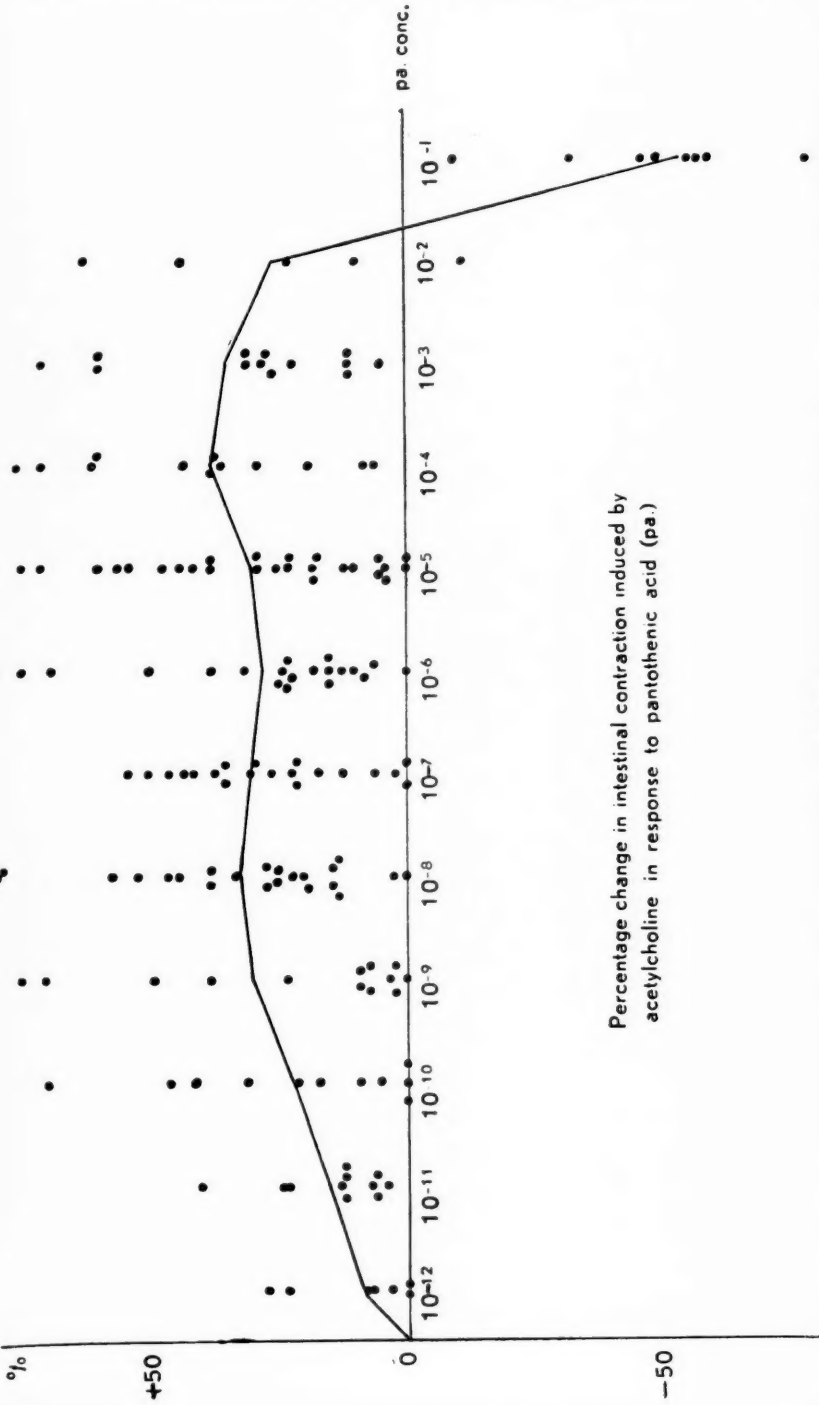


Fig. 1.

0.1 to 5 micrograms of acetylcholine. After recording the contraction produced by this «standard Ach concentration» we recorded the response to different pantothenic acid concentrations, employed simultaneously, shortly before, or after the application of acetylcholine, i.e. that to both pantothenic acid and acetylcholine. Finally, in informative tests we have examined the response to various concentrations of pantothenic acid alone.

After recording the response to each agent the Tyrode bath was exchanged, until the initial resting tone of the intestine was restored.

At the end of the experiment the curves obtained were analysed comparatively.

When the contraction in response to the combined application of pantothenic acid and acetylcholine exceeded that elicited repeatedly by the «standard Ach concentration» previously, the sensitivity of the gut to acetylcholine was considered to be increased; when the opposite took place, a decreased sensitivity was present. In both cases the change is expressed in percentage of the basic contraction (that produced by the standard Ach). (Fig. 1.)

The acetylcholine used was kindly donated by the firm La Roche (Basle), for which we express our thanks also here. The calciumpantothenate solution has been prepared by us.

RESULTS

We have tested pantothenic acid in the concentration range of from 10^{-13} to 5×10^{-1} for effect on the acetylcholine-sensitivity of the isolated intestine. As it is visible also in the Table, the following results have been obtained.

1. Physiological concentrations (10^{-2} to 10^{-12}) of pantothenic acid increased the acetylcholine-sensitivity of the intestine by an average of about 30 per cent. This increase is only partly proportionate to the concentration of pantothenic acid employed; from 10^{-12} to 10^{-9} there is a rising tendency, in the range of from 10^{-9} to 10^{-3} there is a rather constant high level, a decline begins at 10^{-3} and the effect ceases under the 10^{-2} concentration.

2. Pantothenic acid concentrations higher than the physiological (5×10^{-2} and higher) definitely diminish the acetylcholine-sensitivity of the intestine. Thus, the initial effect is inverted.

3. When used in concentrations lower than the physiological

(10^{-12} and lower), pantothenic acid has no influence on the acetylcholine-sensitivity of the isolated intestine.

4. All these effects are independent of the order in which pantothenic acid and acetylcholine are employed.

In the informative tests pantothenic acid alone (in concentrations from 10^{-1} to 10^{-10}) caused only minor contractions in the isolated intestine.

DISCUSSION

Only scanty evidence as to the effect of pantothenic acid on the acetylcholine-sensitivity of the isolated intestine has been published before our investigations. In his dissertation Chaves Castanos (6) mentions that in the isolated guinea pig intestine pantothenic acid prolongs or enhances the action of acetylcholine. Recently, Mosler and Vorherr (31) have made a similar observation; they report that pantothenic acid enhances the submaximal contractions elicited by acetylcholine in the isolated guinea pig gut. They emphasize at the same time that several organic acids other than pantothenic acid (dl-serine, alpha-ketoglutaric acid, malonic acid, alanine, etc.) have a similar action. Also, they think it to be remarkable that pantothenic acid increases the intestinal action not only of acetylcholine, but also of choline, histamine and nicotine. From the latter observation, as well as from the fact that morphium inhibits the slight peristalsis-increasing action of pantothenic acid, but not that of acetylcholine, it is concluded that the site of action of pantothenic acid is not identical with that of acetylcholine.

Other experimental data relate to the increase of peristalsis in response to pantothenic acid alone, without making definite statements as to the site and mechanism of action. For example, minor or major intestinal contractions have been produced by the use of pantothenic acid in man, at operation (21), in rat and guinea pig intestine (7) and in rabbit gut in vitro (32). Nassi (32) mentions that the contraction was proportionate to the concentration of pantothenic acid, but when it exceeds the physiological, a decrease rather than an increase takes place. We can confirm these observations: as it has been mentioned, in our experiments the use of pantothenic acid concentrations exceeding the physiological resulted in a decrease of the acetylcholine-sensitivity of the intestine

and in a diminution of the contraction caused by acetylcholine. Nassi's statement is in agreement also with the practical finding that in paralytic ileus the therapeutic effect of pantothenic acid cannot be enhanced by an unrestricted increase of the dose (1, 25, 49).

Thus, while it is generally accepted today that pantothenic acid enhances intestinal peristalsis, there is a considerable divergence of opinion as to the site and mode of action. There are two principal trends:

One group of the investigators (and this is the larger group) thinks that irrespective of the presence or absence of deficiency, pantothenic acid exerts a pharmacodynamic action on the intestine; they claim that the site of action of pantothenic acid is coenzyme A or acetylcholine, into which it is built (29).

Others suggest that pantothenic acid enhances intestinal peristalsis exclusively in deficiency states and believe the action to be a substitutive one. These authors claim that pantothenic acid has a site of action other than acetylcholine (31) (Adrenal cortex? Other, unknown site of action?).

We think that our experiments on the intestine of animals not suffering from pantothenic acid deficiency may narrow the gap between the two views, because on the one hand they confirm the pharmacodynamic nature of the intestinal effect of pantothenic acid, and, on the other hand, indicate that pantothenic acid acts not only through coenzyme A or acetylcholine, but also by increasing the sensitivity of the intestine to acetylcholine (change in membrane potential?).

Finally, we should like to point out that also aneurin, an other factor of the vitamin B complex, has been shown to increase the peristalsis (16) and the acetylcholine-sensitivity (47) of the rat intestine *in vitro*. In this connection reference should be made also to the observation that aneurin, like pantothenic acid, is involved in the acetylation of choline as well (53).

SUMMARY

In physiological concentrations (10^{-2} — 10^{-12}) pantothenic acid increases the sensitivity of the isolated rat intestine to acetylcholine. Higher concentrations decrease, lower ones do not influence this

sensitivity. This observation supplies new evidence as to the mechanism of action by which pantothenic acid stimulates intestinal peristalsis.

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INCORPORATION OF ACETATE- ^{14}C INTO SKIN COMPONENTS

by

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Below I present preliminary experiments designed to answer the following problems: (a) how the acetate incorporation compares with the incorporation of glycine to the collagen fractions (2), (b) how the keratin compares with collagen and (c) which is the relation of skin albumin and globulin to the plasma proteins (6). Earlier an investigation has been made on the synthesis of collagen fractions (5). Even if the problem is complicated by the differences in the formation of the amino acids, this approach affords a clarification on this side of acetate metabolism.

METHODS

Treatment of Animals. — Five male and female albino rabbits were used. Their weights at the time when radioactive acetate was given were between 810 and 1,040 g. The animals were starved before injection for two days, but were allowed free access to water. After injection they were fed *ad libitum* normally. Radioactive acetate ($\text{CH}_3^{14}\text{COOH}$, specific activity $31.7 \mu\text{c}/\text{mg}$) was injected subcutaneously into the back in 1 ml of 1% $\text{CH}_3\text{COO Na}$; the dose injected was 19–25 $\mu\text{c}/100 \text{ g. body wt.}$

The rabbits were killed under ether anesthesia by opening the thorax four, seven or nine days after injection. The blood (about

15—30 ml) was removed by cardiac puncture. The weights of the rabbits after killing were 970—1,060 g. and increasing of wt. was 30—41 g/day/animal. The skins were stripped and stored at —15°C.

Fractionation of Skin Proteins. — The hairs and subcutaneous fat of the skins were removed mechanically. Between 55 and 80 g. of skin were obtained from a rabbit. The skins were ground in a mincer and suspended in 0.2 M Na_2HPO_4 (pH 9) at 4°C. The steps of fractionation are seen in Fig. 1. Extractions were done either with a mechanical shaker or with the aid of stirring. The procedure adopted was a modified form of that described by Harkness *et al.* (2). All extractions were carried out at 4°C.

The solids obtained from the dialysis of the phosphate soluble fraction against water were fractionated no longer because of the small amounts. All the fractions were washed with acetone and ether.

Fractionation of Serum Proteins. — The serum proteins were fractionated to albumin and globulin fractions with disodium sulfate according to Kibrick and Blonstein (4). The fractions were then dialysed against water and washed with acetone and ether.

Measurement of Radioactivity. — The various protein fractions were hydrolyzed by heating with 6 N-HCl for 16 hr in sealed tubes in an oven kept at 105°C. The hydrolysates were concentrated to dryness in steam bath and the residue taken up in water. Humins and possible salts were removed by Dowex 50. Finally the samples concentrated to dryness on the disks under the infra red lamp. The measurement of radioactivity was carried out on solid samples placed on disks of identical geometry under conditions of infinite thickness by end window Geiger-Müller tube (TCG-2; Tracerlab).

RESULTS

The results are given in Table 1. There are differences between the individuals, presumably due to the resorption. The ratio albumin/globulin in the skin and serum is similar but the absolute activities are higher in serum. The activities of the keratin and collagen are of similar magnitude, as can be expected in a fast growing animal. The activity in the insoluble collagen is remarkably high. Therefore a contamination of acetylated mucopoly-

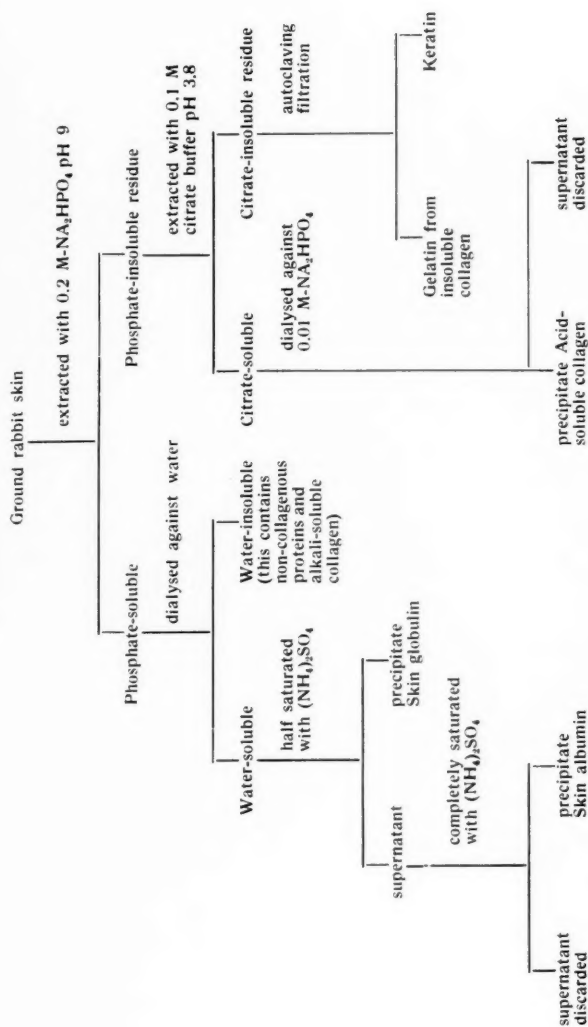


Fig. 1. — The fractionation of skin proteins of rabbits.

TABLE 1

THE RADIOACTIVITY (C.P.M.) OF INFINITELY THICK SAMPLES FROM RABBIT SKIN AFTER INJECTION OF $\text{CH}_3^{14}\text{COOH}$ CORRECTED FOR INCREASE IN BODY WEIGHT AND REDUCED TO A DOSE OF 25 $\mu\text{C}/100$ G. BODY WEIGHT

Fraction	Days after injection				
	4	7		9	
		a	b	c	
Skin					
albumin	..	38	22	..	11
globulin	..	46	113	..	15
water-insoluble proteins	..	38	77	33	13
acid-soluble collagen	..	44	29
insoluble collagen	52	29	78	16	19
keratin	83	34	64	30	16
Serum					
albumin	58	49	..	45	29
globulin	83	59	..	55	33

saccharides might be suspected. The results show that, if Neuberger's result of 6—8 days equilibration time (6) is correct, the resynthesis of plasma albumin is so rapid that a difference persists.

DISCUSSION

The previous studies on the metabolism of the rabbit skin proteins show that activity of skin albumin never exceeded 65 per cent of that of plasma albumin. The equilibration between plasma and skin albumin is reached in about 6—8 days (6). According to my results the ratio skin albumin/serum albumin is the same magnitude and this same ratio is between skin globulin and serum globulin, too. It is possible that skin albumin may be derived from the same pool as serum albumin but a part of skin albumin is generated from another pool. That is same to skin globulin. We see also that the activity of globulin is always higher than that of albumin due to the greater turnover of albumin (1).

Harkness *et al.* (2) have earlier studied the incorporation of glycine to the rabbit skin proteins. There were no greater differences in various skin components after the fourth day as also Jackson (3) has indicated in granuloma induced by carrageenin. In my results there are no remarkable differences in skin components after seven

days. The activity of alkali-soluble collagen is, however, surprisingly high in the seventh day. This is due to non-collagenous contaminants. Samples taken at 1—4 day range would have given better information.

The activity of keratin is higher than expected. Presence of other proteins in denatured state is not quite excluded.

SUMMARY

The incorporation of radioactive acetate into skin components was studied. After the seventh day there are no remarkable differences between various collagen fractions. The activity of skin albumin and skin globulin is lower than that of serum albumin and serum globulin. The activity of albumin is lower than that of globulin both in skin and in serum.

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PROTEOLYTIC ENZYMES OF SYNOVIAL FLUID

WITH SPECIAL REFERENCE TO INTRA-ARTICULARLY APPLIED
HYDROCORTISONE

by

TEPPO VARTIO

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Various substances and properties of the synovial fluid have been examined, *e.g.* electrolytes (2, 6, 16, 17), proteins (2, 3, 5, 6, 8, 11, 12, 17), hyaluronic acid (1, 3, 6, 9, 11), viscosity (6, 9, 11, 12, 17), and colloid osmotic pressure (3, 6, 11). The effect of intra-articularly applied corticosteroid hormones on the pathological synovial fluid has been thoroughly studied (3, 4, 6, 7, 12, 17). The abatement of inflammatory symptoms from the joint and decrease in the joint effusion have been the generally perceived effects of these hormones. In the following a study is made of the appearance of the three proteolytic enzymes, pepsin, cathepsin, and trypsin, in the synovial fluid in various pathological conditions, and the influence of hydrocortisone upon the enzymes.

MATERIAL AND METHODS

The samples of the synovial fluid were obtained from the knee joint by puncture of 14 patients suffering from rheumatoid arthritis, 6 patients with osteoarthritis (degenerative arthritis, osteoarthritis), and 12 patients with traumatic hydarthritis. From the same sample the three proteolytic enzymes, and if the sample was enough, also leukocytes and total proteins, were examined. The pepsin, cathepsin and trypsin were determinated according to the method described

earlier (15). The determinations were made in duplicates and in some cases in triplicates. In 11 cases, after the prove had been drawn into the syringe, 50 mg. of hydrocortisone acetate (Hydrocortone Acetate Suspension, Merck) was injected into the joint cavity during the same puncture, and a second puncture for the determinations was performed 24 hours later in 4 cases, 2 days later in 4 cases, 3 days later in 2 cases, and 5 days later in one case.

The Accuracy of the Pepsin, Cathepsin and Trypsin Determination

Methods: When checking the accuracy of the methods in double and in some triple determinations, the accuracy for the pepsin determination was found to be $\pm 2 \times 0.26$ mg edestin/ml, for the cathepsin method $\pm 2 \times 0.08$ mg edestin/ml, and for the trypsin method $\pm 2 \times 0.18$ mg protein/ml, if the determinations were made in duplicates.

RESULTS

The results of the three proteolytic enzyme determinations appear from table 1. From the three proteolytic enzymes the pepsin seemed to exist in greater amounts than the other two of the specimens. Thus the result in this experiment agrees with that obtained in the examination of the three enzymes in cerebrospinal, pleural, and peritoneal fluids (13, 14). From the different diseases the pepsin and cathepsin seemed to appear in greater amounts in the specimens of osteoarthritis and traumatic hydrarthrosis than in those of rheumatoid arthritis. The greatest difference was between the pepsin values in osteoarthritis and in rheumatoid arthritis (means 1.57 ± 0.40 mg edestin/ml corr. 0.84 ± 0.16 mg edestin/ml). This difference was not, however, statistically significant, which could be due to the small number of cases. No definite dependance of the enzyme values on the leukocytes or the total proteins in the specimens could be found.

In table 2 the influence of the intra-articularly applied hydrocortisone on the proteolytic enzymes of the synovial fluid is expressed. As it appears from the table, this hormone caused no definite change in the enzyme pattern.

TABLE 1

PEPSIN, CATHEPSIN AND TRYPSIN IN SYNOVIAL FLUID IN VARIOUS DISEASES

Age	Sex	Pepsin mg Edestin/ml	Cathepsin mg Edestin/ml	Trypsin mg Protein/ ml	Leukocytes				Proteins
					Amount	Granul.	Lymphoc.	Monoc.	
<i>Arthritis rheumatoides:</i>									
40	♀	0.94	0.21	0.42	++	95	5	0	5.7
17	♀	0.06	0.30	0.07	++	55	25	20	4.9
46	♀	0.77	0.40	0.27	+++	95	5	0	5.8
18	♀	0.70	0.07	0.05	++	70	20	10	—
16	♀	1.00	0.65	0.62	+++	90	10	0	5.8
20	♀	0.25	0.15	0.17	+++	90	10	0	5.0
30	♀	1.70	0.55	1.07	++	85	15	0	—
45	♀	0.35	0.10	0.00	—	—	—	—	—
22	♀	0.32	0.00	0.00	+++	20	80	0	—
45	♀	0.63	0.15	0.07	—	—	—	—	—
57	♀	0.25	0.30	0.20	++	95	5	0	—
61	♀	1.32	0.12	0.12	—	—	—	—	—
28	♂	1.50	0.42	0.00	++	2	87	11	4.5
21	♂	2.00	1.20	0.00	++	38	35	27	3.9
Mean		0.84 ± 0.16	0.33	0.22					
<i>Arthrosis deformans (osteoarthritis):</i>									
80	♀	2.65	1.10	0.20	—	—	—	—	—
75	♀	2.85	0.12	0.50	+	50	28	22	3.8
61	♀	1.00	0.00	0.27	++	10	80	10	4.1
55	♀	1.20	0.45	0.00	++	75	25	0	4.1
54	♀	0.35	0.00	0.60	—	—	—	—	—
70	♂	1.40	2.40	0.15	+++	95	5	0	5.1
Mean		1.57 ± 0.40	0.67	0.28					
<i>Hydrops genu traumatica:</i>									
23	♀	0.20	0.10	0.17	++	90	10	0	—
31	♀	1.50	0.37	0.00	+	15	60	25	3.7
47	♀	0.09	0.12	0.00	+	—	—	—	3.3
24	♀	0.80	0.35	0.42	—	—	—	—	—
24	♂	3.65	2.27	0.00	—	—	—	—	—
27	♂	3.20	2.70	0.00	—	—	—	—	—
25	♂	0.20	1.05	0.35	++	80	15	5	4.3
36	♂	1.50	0.97	1.10	—	—	—	—	—
52	♂	—	2.00	0.02	++	75	25	0	5.1
30	♂	2.15	0.32	0.17	+	—	—	—	4.3
28	♂	0.31	0.00	0.00	+++	56	34	10	5.9
59	♂	1.50	0.45	0.47	—	—	—	—	4.1
Mean		1.37 ± 0.37	0.89	0.22					

+ = some leukocytes
 ++ = moderately leukocytes

+++ = plenty of leukocytes
 — = not examined

TABLE 2
 INFLUENCE OF INTRA-ARTICULARLY APPLIED HYDROCORTISONE ON PROTEOLYTIC ENZYMES OF SYNOVIAL FLUID

Age	Sex	Pepsin mg Edestin/ml		Cathepsin mg Edestin/ml		Trypsin mg Protein/ml		Leukocytes after Hydroc. Diminished \div Increased $+$ Not Particularly Changed \pm	Proteins %	
		Before Hydroc.	1—5 Days after Hydroc.	Before Hydroc.	1—5 Days after Hydroc.	Before Hydroc.	1—5 Days after Hydroc.		Before Hydroc.	1—5 Days after Hydroc.
<i>Arthritis rheumatoides:</i>										
30	♀	1.70	1 : 1.60	0.55	2.50	1.07	1.50	+	4.5	5.0
16	♀	1.00	1 : 0.20	0.65	0.17	0.62	0.15	\div	5.8	—
			2 : 1.10	0.40	0.40		0.40	\pm	—	4.8
17	♀	0.06	1 : 0.14	0.31	0.19	0.07	0.26	+	4.9	4.7
40	♀	0.94	1 : 2.40	0.20	0.06	0.42	0.00	—	5.7	—
46	♀	0.77	2 : 0.72	0.40	0.15	0.27	0.57	\div	5.8	4.6
18	♀	0.70	2 : 1.45	0.07	0.57	0.05	0.00	\pm	5.1	—
45	♀	0.63	2 : 0.40	0.15	0.25	0.07	0.12	—	—	—
61	♀	1.32	3 : 1.25	0.12	0.15	0.12	0.00	—	—	—
<i>Arthrosis deformans (osteoarthritis):</i>										
61	♀	1.00	2 : 2.20	0.00	0.42	0.27	0.00	\pm	4.1	4.4
<i>Hydrops genu traumatica:</i>										
23	♀	0.20	3 : 0.00	0.10	0.00	0.17	0.00	—	—	—
30	♂	2.15	5 : 0.00	0.32	0.06	0.17	0.00	\pm	4.3	4.4
Mean		0.95	0.95	0.26	0.41	0.30	0.25			

— = not examined

COMMENTS

The amount of the three proteolytic enzymes in the synovial fluid didn't seem to have any relation to the amount of leukocytes or total proteins in the fluid. No fractionation of proteins, however, was performed in this study. As the proteolytic enzymes are known to be like albumins, they could be in relation to the albumin content of the fluid. In the osteoarthritic patients the albumin content of the synovial fluid has been found to be higher than in the rheumatoid arthritic patients (5). This could thus explain the higher amount of proteolytic enzymes in the osteoarthritic patients found in this study.

The proteolytic enzymes of the synovial fluid are in all probability derived from blood. The great variability of these enzymes in the blood (10, 15) depending, among other things, on the occasional state of their gastric source, could thus also declare the great variability of these enzymes in the synovial fluid found in this study.

SUMMARY

The three proteolytic enzymes, pepsin, cathepsin, and trypsin, were studied from the synovial fluid of patients suffering from rheumatoid arthritis, osteoarthritis, and traumatic hydrarthrosis. Variable amounts of these enzymes were found in the specimens and from these the pepsin seemed to exist in greater amounts than the other two. From the different diseases the pepsin and cathepsin seemed to appear in greater amounts in the specimens of osteoarthritis and traumatic hydrarthrosis than in those of rheumatoid arthritis. The greatest values on an average were the pepsin values in the osteoarthritis. The intra-articularly applied hydrocortisone seemed to cause no definite change in the enzyme pattern.

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CIRCULATORY RESPONSE OF CAT TO MALE FERN SUBSTANCES ¹

by

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(Received for publication December 1, 1959)

In a previous study, the negative inotropic and chronotropic effects of N-methylglucaminates of male fern extract and flavaspidic acid on isolated heart muscle preparation have been observed (5). Similar results were obtained with suspensions of each substance separately (3). The changes in the function of the heart muscle alone give only partial information of the response of the circulatory system in the living animal. Circulatory studies in connection with injections of male fern extract and flavaspidic acid glucamate were performed in the present series.

METHOD

Fifteen cats weighing 1.8 to 6 kg were used as experimental animals. Intraperitoneal Nembutal anesthesia (40 mg per kg) was given. A Lehman catheter No. 5 was inserted into aorta through the femoral artery for pressure recording. The right ventricular pressure was recorded through a catheter No. 6 inserted through the external jugular vein. The test substance was injected through a cannula into the femoral vein with the aid of a Palmer slow injection apparatus.

Blood pressures were recorded by two Sanborn manometers.

¹ Aided by grants from Leiras, Pharmaceutical Manufacturers, and from the Suomen Kulttuurirahasto.

The tracings were obtained simultaneously with the electrocardiogram (extremity lead II) by means of a Sanborn 4-channel recorder.

N-methylglucamines of male fern extract and of flavaspidic acid dissolved in 1 per cent water were used and injected with a constant speed of 1.43 ml per minute until the animal died. The preparation of the test substances has been previously described (5).

RESULTS

One of the most important features was the response of the *aortic pressure*. Both the systolic and diastolic pressures remained unchanged while changes in the right ventricular pressure and in the electrocardiogram had already been observed. At the end of the experiments, the aortic pressure dropped suddenly, indicating fatal damage to the test animal. The fall in the aortic pressure was soon followed by stopping of the heart beating and respiration. As Figs. 1 and 2 show the pressure fall occurred while the last fourth of the injection was given. No essential differences were observed between the various test substances. Fig. 3 shows the final abrupt pressure changes in one experiment.

The lethal doses of the test substances were determined on the basis of the blood pressure fall. This was on an average 115 mg per kg (range 61—146 mg per kg) in the male fern extract series and 93 mg per kg (54—149) in the flavaspidic acid series.

The *right ventricular pressure* increased in all experimental animals (Figs 4 and 5). The increase began immediately after the start of injection and reached the maximum when half of the lethal dose had been injected. Then the right ventricular pressure decreased and was at its initial value when the sudden decrease in the aortic pressure began. After this phase the right ventricular pressure decreased abruptly. A sample of original pressure curves is seen in Fig. 6, recorded before and during injection of male fern extract glucamate.

The *electrocardiogram* showed various gradually developing changes. In the flavaspidic acid series, the T-wave changes appeared when $\frac{1}{5}$ — $\frac{1}{3}$ of the substance had been injected. Decrease, increase, inversion or disappearance of T occurred in various cases and phases. T-wave changes in the male fern extract series occurred comparatively late, in every second case not until in connection with the last occurrences before death.

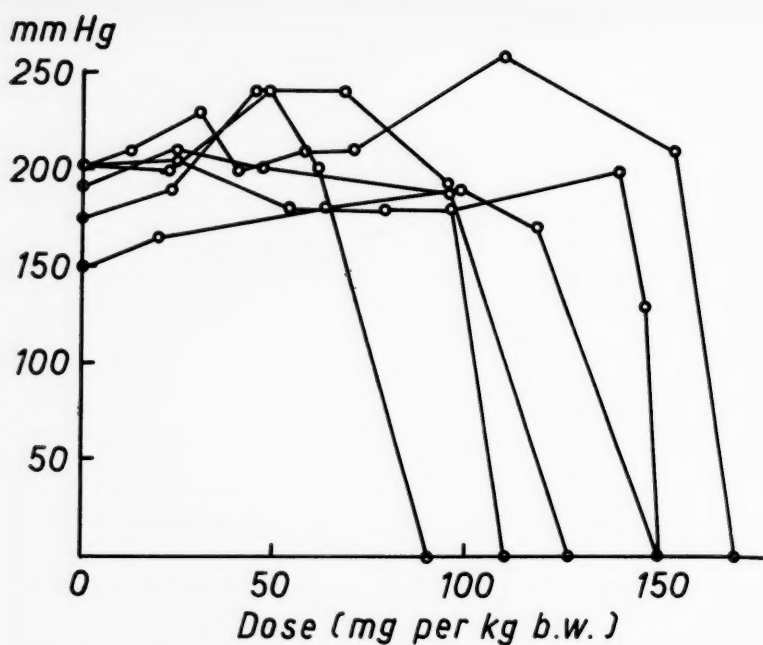


Fig. 1. — The systolic aortic pressure during intravenous administration of N-methylglucamine of male fern extract.

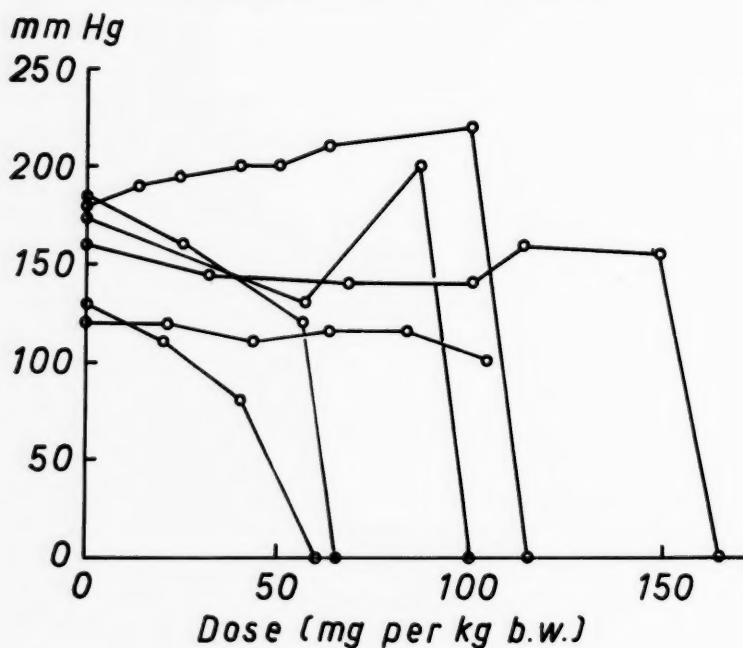


Fig. 2. — The systolic aortic pressure during intravenous injection of N-methylglucamine of flavaspidic acid.

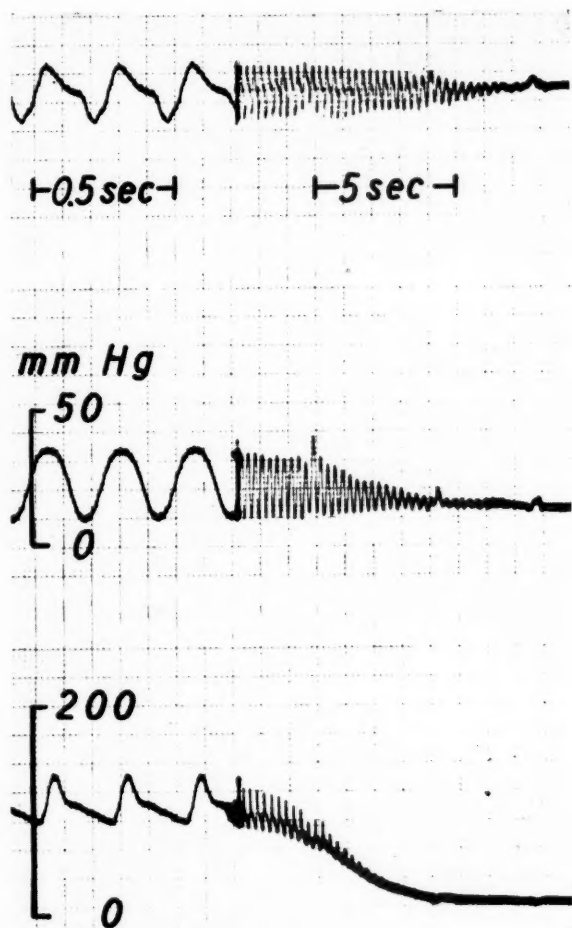


Fig. 3. — Changes in the aortic pressure (lower curve), right ventricular pressure (middle curve) and electrocardiogram (upper curve) before death. Cat, 2.8 kg. Total dose 410 mg of male fern extract glucamate.

Diminishing of all electrocardiographic deflections was characteristic of the animals treated with male fern extract. This appeared when half of the total dose had been injected. The change occurred only once in the flavaspidic cats.

During the last third or fifth of the experiment, prolongation of QRS interval, diminishing of QRS-voltage and disappearance of P-wave were common in both series. Before or simultaneously

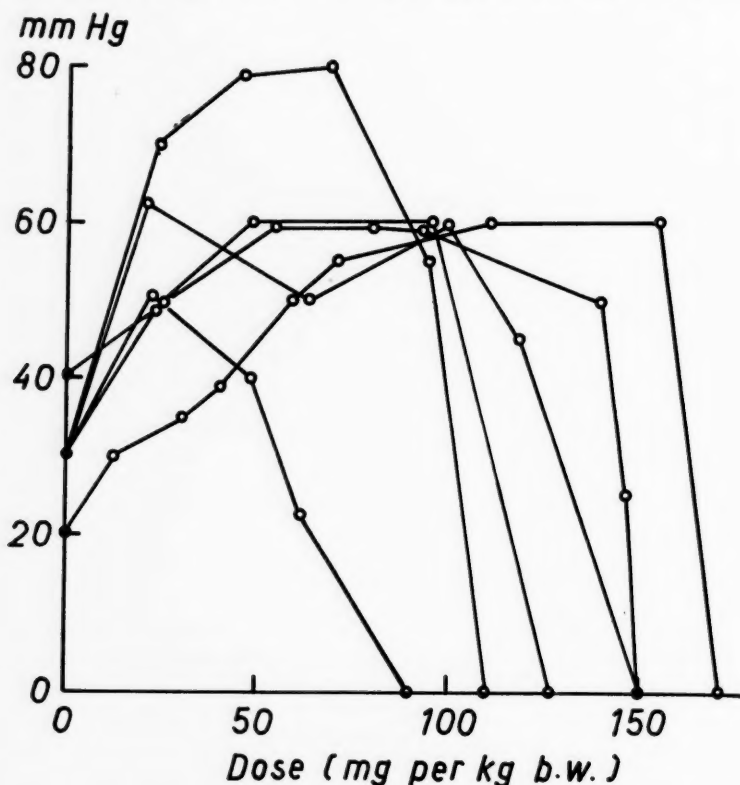


Fig. 4. — The right ventricular pressure during intravenous administration of N-methylglucamine of male fern extract.

with the final drop in aortic pressure, the electrocardiogram lost its usual appearance. (Figs. 3 and 7).

Atrio-ventricular block, ST-deformations and arrhythmic contractions occurred only in sporadic cases without any regularity. Development of the electrocardiographic changes in an experiment with male fern extract glucamine is seen in Fig. 7.

No essential heart rate changes were observed during the first half of the tests. At the end, the rate decreased in all animals.

Control tests with distilled water injections were performed in two cats. Fig. 8 shows pressure and electrocardiographic recordings before and after injection of 80 ml of water. Heart rate retardation was the only essential change.

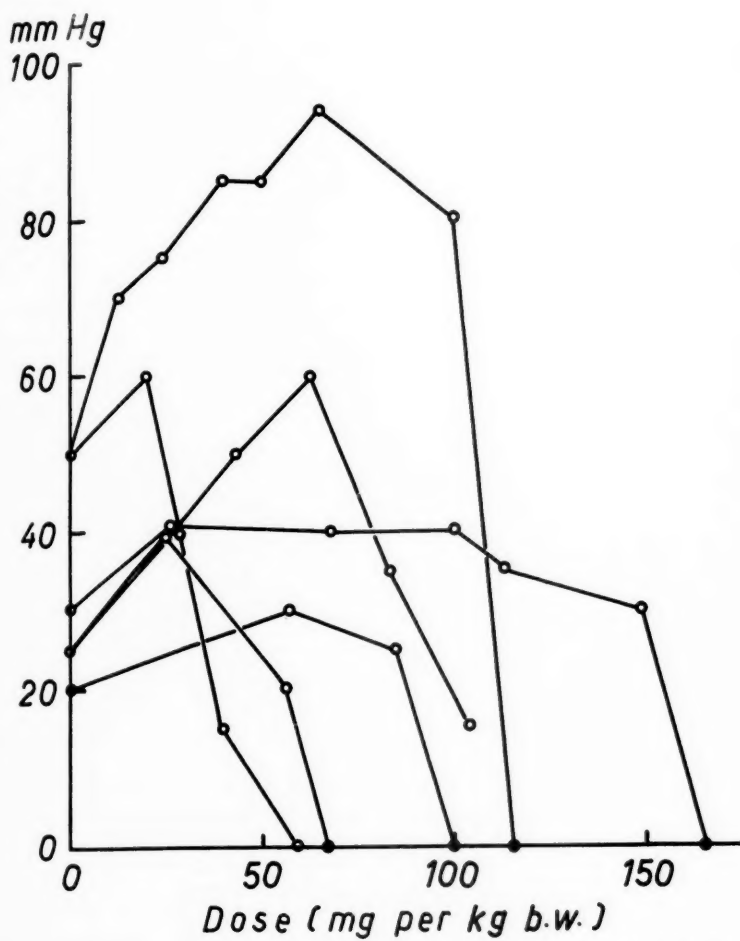


Fig. 5. — The right ventricular pressure during intravenous injection of N-methylglucamine of flavaspodic acid.

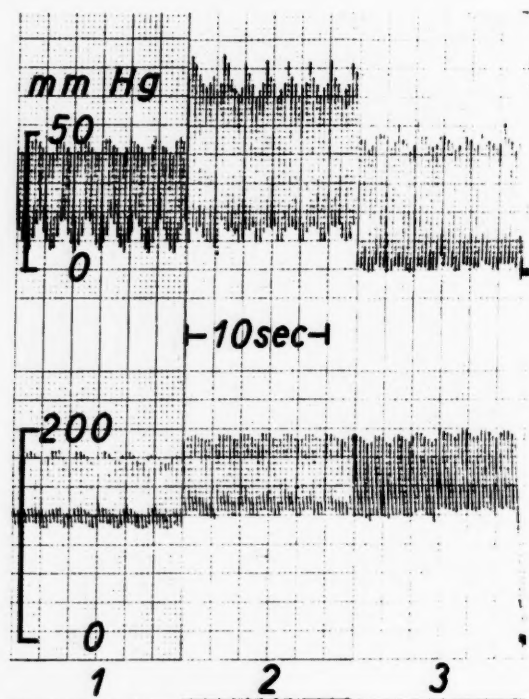


Fig. 6. — Aortic (lower) and right ventricular (upper) pressure curves of the same animal as in Fig. 3. (1) before male fern extract glucamine injection, (2) after injection of 70 mg and (3) 380 mg of the test substance. Note the increase of the right ventricular pressure.

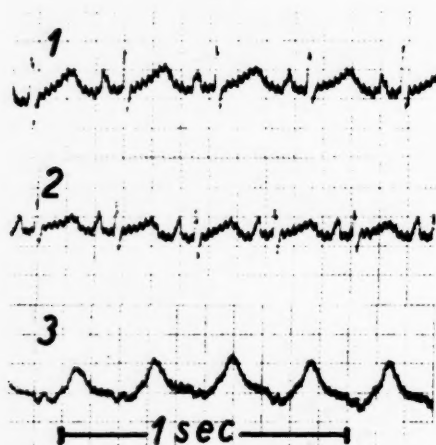


Fig. 7. — Electrocardiogram of a 5 kg cat (1) before the test (2) after injection of 300 mg of male fern extract glucamine, and (3) after 620 mg. The lethal dose was 650 mg. Note the diminution of the deflections of the ventricular complex in tracing 2.

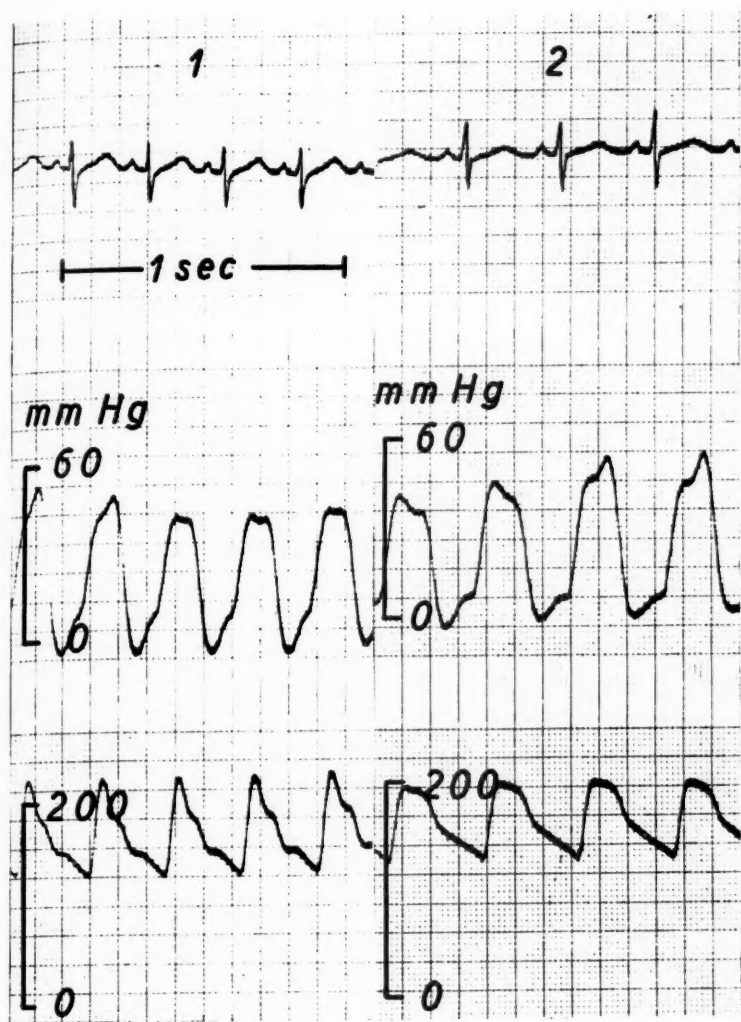


Fig. 8. — Electrocardiogram (upper curve), right ventricular pressure (middle curve) and aortic pressure (lower curve) of a 3.4 kg cat before (1) and after (2) injection of 80 ml of distilled water during 1 hour.

DISCUSSION

The most interesting finding was probably the increase of the right ventricular pressure following upon administration of N-methylglucaminates of male fern extract and of flavaspidic acid. This change occurred without any alterations in the aortic pressure. The pressure increase in the right ventricle was apparently due to increased resistance in the pulmonary circuit. The test solutions had an opalescent cloudy appearance and one might think that undissolved particles had mechanically occluded the pulmonary capillaries. This idea is less probable, however, because the aortic pressure remained unchanged. When the pulmonary capillaries become occluded by injecting india ink or starch particles, the aortic pressure falls immediately (2). If male fern extract and flavaspidic acid were to increase the pulmonary blood pressure when used therapeutically, the cardiac complications would be easier to understand.

No specific changes were seen in the electrocardiogram. Alterations in T-wave and ST-interval are the most common observations in the patients treated with male fern extract (1). In the present study various T-wave changes appeared somewhat earlier in the flavaspidic acid series than in the male fern extract tests. Isolated ST-interval changes were not a regular phenomenon. Electrocardiographic alterations point to diffuse myocardial damage. Intraventricular conduction retardation and other terminal changes were similar to those observed in many other experimental conditions leading to death of the test animal. In any case the heart stopped effective beating before termination of respiration and in agreement with Oelkers and Ohnesorge (4) which shows that heart damage was the essential cause of death. When the effects of male fern extract and flavaspidic acid glucaminates were compared, no essential differences were found between these two substances in the present material.

SUMMARY

The effect on the circulation of intravenously injected N-methylglucaminates of male fern extract and of flavaspidic acid was studied in cats. Both substances were found to increase the right

ventricular pressure. The aortic pressure remained unchanged until the sudden terminal drop.

In the electrocardiogram, signs of diffuse myocardial damage without any regular specific changes were observed.

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MITOTIC ACTIVITY IN THE EPITHELIUM OF THE SMALL BRONCHI OF MICE WITH A HIGH INCIDENCE OF SPONTANEOUS LUNG TUMOURS¹

by

HARRY BJÖRK and MATTI HÄRKÖNEN

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Certain strains of laboratory mice are high in the incidence of lung tumours, particularly of the type adenoma-adenocarcinoma. In the monograph »Biology of the Laboratory Mouse» these tumours are described in detail, and it is stated — among other observations — that they are derived from the lining cells of the bronchi and the alveoli (2).

It seemed of interest to try to determine whether any unusual mitotic activity can be demonstrated in the lungs of mice of high tumour strains but not yet of the age when lung tumours begin to appear in a significant percentage of animals. On the basis of the statement referred to above it seemed possible to make the mitotic counts on bronchial or alveolar tissue. Using the latter, mitotic counts are more exacting technically, and it is thus more difficult to obtain reliable results. For the purpose of our studies we therefore chose the small bronchi.

Two different strains of mice supplied by the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, U.S.A., were used for the study. One strain, A/Jax, has a high incidence of spontaneous lung tumours, while the other, C57, is not known to develop such tumours and thus is well suited for control purposes.

¹ This investigation was supported by the Damon Runyon Memorial Fund (291—D).

The Jackson laboratory sent us the following information regarding the A/Jax strain:

Approximately 63% of the males and 70% of the females develop spontaneous lung tumour at the end of normal life span. The biological property is primary adenoma and the number increases with advancing age and onset can be hastened with some type of application of carcinogen. The following figures were also obtained concerning the incidence of animals with lung tumours at various ages: 3 months 2.5 per cent, 6 months 9.3 per cent, 9 months 34.5 per cent (4).

METHODS

Thirty animals of the strain A/Jax and 29 of C57 were examined at the age of 3 months. In the case of both strains, body weight was from 18 to 25 gm.

The animals of both strains were divided into two groups. One was studied without exposure to mitotic poisons, in the other colchicine was given subcutaneously as a dose of 0.09 mg. This dose has been found to be the largest possible to be used subcutaneously in mice in experiments of about 24 hours duration (1).

The animals were killed by decapitation in batches of five, the time of exposure to colchicine being 5, 12 and 29 hours. Bouin's solution was used for fixation of the lungs. The preparations were stained with hematoxylin-eosin. The number of mitoses was counted per 3000 epithelial cells. Student's *t*-test was used for statistical analysis.

RESULTS

The average frequency of mitoses per 1000 cells for the groups and subgroups of each strain is given in table 1.

The table shows that the untreated A/Jax mice had by no means a higher mitotic activity than the corresponding mice of the strain C57. On the contrary, the average for the latter (0.55) was higher than for the former (0.29). Considering the nature of the experiments, this difference is not statistically significant ($t = 1.486$).

After colchicine injection the mitotic numbers were higher for

TABLE 1
AVERAGE FREQUENCY OF MITOSES PER 1000 EPITHELIAL CELLS OF THE SMALL BRONCHI IN UNTREATED AND COLCHICINE-TREATED MICE WITH (A/JAX) AND WITHOUT (C57) A HIGH INCIDENCE OF SPONTANEOUS LUNG TUMOURS

Group	Number of Animals per Group	Average Frequency per Group	Average Frequency per Subgroup		
			a)	b)	c)
I Untreated					
A/Jax	15	0.29	0.44	0.30	0.12
C 57	15	0.55	0.68	0.78	0.18
II Colchicine 0.09					
A/Jax	15	1.01	0.74	1.38	0.92
C 57	14	1.03	1.00	1.65 ¹	0.56

a) Killed 5. 10., 3—4 p.m. (exposure to colchicine 5 hours)

b) " 5. 10., 10—11 p.m. (" " " 12 ")

c) " 6. 10., 3—4 p.m. (" " " 29 ")

¹ This subgroup consists of 4 animals, all others of 5 animals.

both of the strains studied, and even in each subgroup, than for the untreated animals, yet, statistically, the difference is not very marked. Because of the small number of the animals it seemed advisable to disregard the subgroups in the statistical analysis and take only the groups into account. As far as C57 is concerned, the difference in the above respect between the treated and the untreated animals was not statistically significant ($t = 1.702$), although the difference between the individual figures is distinct enough. The strain A/Jax, however, showed a significant difference ($t = 2.769$, $P > 0.01$). In spite of this the two strains did not differ significantly in mode of reaction to colchicine ($t = 0.600$).

Despite the absence of statistically significant results in the case of strain C57, the regularity of the results in all subgroups indicates that the experimental mice of both strains had responded to colchicine. This is also corroborated by the types of mitoses found: typical so-called colchicine mitoses occurred in the sections prepared from colchicine-treated animals of both strains.

The response to colchicine showed similar features in the case of both strains: the highest rate occurred 12 hours after injection. Thus, the mode of reaction was the same as found by one of us to be typical for the bronchi (1).

DISCUSSION

Studies on 3-month old experimental mice did not reveal a higher degree of mitotic activity in the small bronchi of strain A/Jax having a high incidence of spontaneous pulmonary tumours than in strain C57, which is not known to develop such tumours. If any increased mitotic activity in the small bronchi occurs at any early age in mice predisposed to lung tumours, this should appear in 3-month old mice since in exceptional cases lung tumour already develops at this age (4).

The above described negative result of our studies may possibly be accounted for by the fact that the A/Jax mice used by us lacked the predisposition to lung tumours typical of the strain. It has, however, been stated that on inbreeding this predisposition appears with great regularity in successive generations of mice (2). For control purposes we allowed 9 of our A/Jax mice to live to the age of nine months, when they were killed and their lungs examined. It appeared that three of the animals had adenoma in one lung, as was to be expected from the data supplied (4).

A possible explanation of the negative result of our studies is that the lung tumours in the mice do not originate from the tissue examined. Some investigators have raised the opinion that the histogenetic relationship between these tumours and the alveolar epithelium is abundantly demonstrated (3). In the beginning of this article we referred to the monograph «Biology of the Laboratory Mouse» as stating that these tumours may also be derived from the bronchi. It is not possible for us to take a position in this matter.

Three of our animals killed at the age of nine months had developed an adenoma. Microscopic examination showed that even areas close to the tumours were free from any sign of reaction though no tumour capsule could be discerned. This is evidence in favour of a sharply circumscribed, localized process. Our negative results might therefore be explained by the fact that, when the mitotic counts are made at random on different areas of pulmonary tissue, there are only slight chances that the count will include those very areas where tumour tissue will subsequently develop and where altered mitotic activity might perhaps be present even in 3-month old animals. We found isolated areas

showing increased mitotic numbers in the lungs of mice of both the strains examined. It was evidently due to chance that such findings were more numerous among the control animals than among the A/Jax mice, and thus such areas cannot by any means be interpreted as foci of incipient tumour formation.

SUMMARY

The mitotic activity in the epithelium of the small bronchi was studied in 3-month old mice with (A/Jax) and without (C57) a high incidence of spontaneous lung tumours. No statistically significant difference in the mitotic rate or the response to colchicine could be demonstrated. Some possible explanations of the results are discussed.

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THE MOUSE TAIL REACTION INDUCED BY MORPHINE AND THE SEDATIVE ACTION AFTER RESERPINE AND NALORPHINE

by

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(Received for publication December 14, 1959)

INTRODUCTION

It has been shown that reserpine releases norepinephrine and 5-hydroxytryptamine from the brain. However it is not certain which of these substances is liberated primarily (1, 5). Morphine liberates norepinephrine from the brain of the cat, too (12), and this can be prevented by the pre-administration of nalorphine (3). The question thus arises as to whether it is possible to effect the morphine-nalorphine antagonism by releasing biological amines in the animal brain with reserpine.

MATERIAL AND METHODS

White mice of the same stock weighing 20—25 g were used. The experiments were performed with groups of 5 mice. A control group receiving morphine alone was used for each experiment.

Drugs and Administration. — Morphine chloride, nalorphine bromide (Burrough's Welcome), reserpine (Serpasil, CIBA), 1-dihydroxyphenylalanine or DOPA (Hoffman la Roche), 5-hydroxytryptamine or 5-HT creatinine sulphate (Vister) and Hydergine (Sandoz) were injected intraperitoneally in various combinations. The substances were dissolved or diluted with water.

Technique. — The mouse tail reaction of Straub-Hermann was used to investigate excitation action induced by morphine. The variations in the strength of the tail reaction were classified 0—3 using the Juul's scale (4).

Each mouse was observed at 15 min. intervals for 75 minutes after the administration of morphine. TD (tail dose) means the morphine dose that causes an average tail reaction of 1.5 according to this scale. It was determined by administering progressive doses of morphine. The changes in TD after different drugs had been administered were studied. Using «normal» TD as a constant the changes in the tail reaction after administering the same drugs were then studied.

To investigate the sedative action of morphine the mice were made to balance on a horizontal glass rod (1.5 cm in diameter) turning on its longitudinal axis 3 times a minute. Normal mice were able to maintain their balance on the rod for several min. but the injection of morphine soon caused them to fall off. The «balance» time was measured with a stop watch. SD (sedative dose) means the morphine dose that causes a fall after an average 30 seconds. The changes in SD after administering different drugs were studied. Using «normal» SD as a constant the changes in the «balance» time after the same drugs were then measured. We tried to differentiate between «true» sedative action and the primary vestibular involvement by careful inspection and by performing each experiment several times.

The T-test was used for the statistical handling.

RESULTS

The optimal dose of nalorphine (5 mg/kg) eliminated the sedative action of morphine very effectively but it affected the tail reaction of morphine poorly and only if injected before morphine. Table 1

TABLE 1.
TD AND SD DOSES AND MORPHINE-NALORPHINE ANTAGONISM AFTER DIFFERENT DRUGS

Drug	Dose mg/kg	Inject. Before Morph.	Morphine Dose mg/kg			Nalorphine (5 mg/kg) Average Preventive Effect %		
			TD	SD	Numb. of Anim.	Tail. React.	Sedat. React.	Numb. of Anim.
Morphine chloride ..			10	25	180	40	100	40
Reserpine	0.1	1.5 h	5	25	40	45	100	35
Reserpine	0.1	4 h	5	20	40	40	95	35
Reserpine	1.0	1.5 h	5	20	45	40	100	35
Reserpine	1.0	4 h	20	15	40	90	35	35
Reserpine	2.0	4 h	20	15	20			
5-HT creat. sulph. ..	5.0	15 min.	10	15	30			
Hydergine	0.05	1.5 h	10	25	45			
Reserpine + Hydergine	1.0							
	0.05	1.5 h	15	15	40			

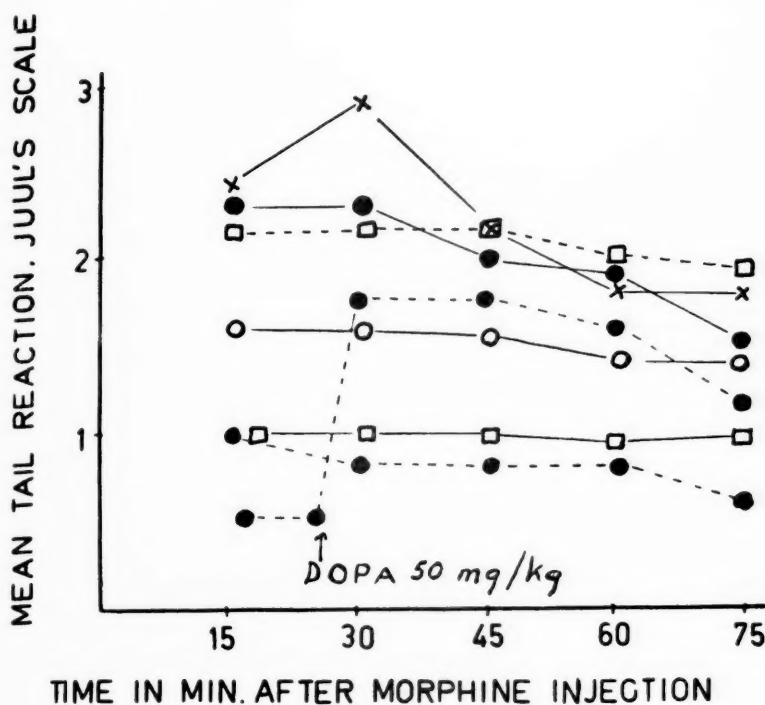


Figure 1. — Changes of tail reaction induced by morphine after some drugs. Morphine dose 10 mg/kg.

- = Morphine
- = Reserpine 1 mg/kg 1.5 hours before morphine injection
- = Reserpine 1 mg/kg 4 h. before morph. inject.
- = Reserpine 1 mg/kg + Hydergine 0.05 mg/kg 1.5 h. before morph. inject.
- = Reserpine 0.1 mg/kg 4 h. before morph. inject.
- x—x—x = DOPA 50 mg/kg 5 min. before morph. inject.

shows the average decrease in tail and sedative reactions (expressed percentually) induced by nalorphine. It also presents the changes in TD and SD after reserpine, 5-HT and Hydergine. Only in great doses (1 mg/kg) does reserpine prevent the mouse tail reaction induced by morphine and when allowed to take effect for 4 hours before the injection of morphine. Then it enhances the antagonistic action of nalorphine. This reserpine dose markedly increases the sedative action of morphine, which is not wholly abolished by nalorphine. After acting for only 1.5 hours this dose of reserpine

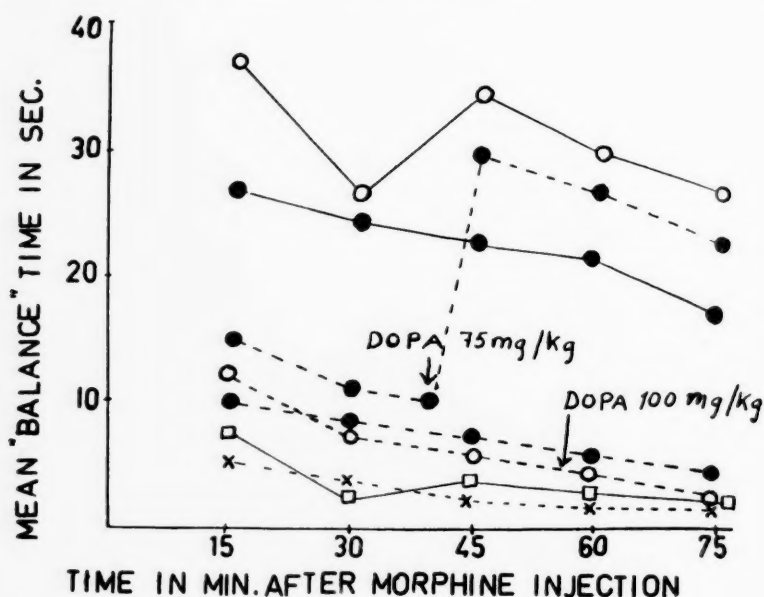


Figure 2. — Changes of sedative action induced by morphine after some drugs. Morphine dose 25 mg/kg.

- = Morphine
- - -○- - -○ = Morphine 30 mg/kg
- = Reserpine 1 mg/kg 1.5 h. before morphine inject.
- - -●- - -● = Reserpine 1 mg/kg 4 h. before morphine inject.
- = Reserpine 1 mg/kg
- +—+—+ = Hydergine 0.05 mg/kg 1.5 h. before morph. inject.
- x- - -x- - -x = 5-HT 5 mg/kg 15 min. before morphine inject.

enhances the tail reaction of morphine significantly (in the T-test $P = 0.025$, fig. 1). Small doses (0.1 mg/kg) have a similar effect that lasts for several hours. Reserpine doses that enhance the tail reaction do not increase the sedative action significantly.

DOPA causes hyperpnoea and piloerection. It even enhances a tail reaction inhibited by a large dose of reserpine (fig. 1). It also decreases the sedative action enhanced by reserpine, but not that induced by morphine alone (fig. 2).

Hydergine itself has no effect upon the tail reaction induced by morphine or upon sedative action but it reverses the stimulating effect of reserpine significantly ($P = 0.03$) (fig. 2) and increases its sedative action (fig. 2).

5-HT increases the sedative action of morphine strongly (fig. 2) but has no effect upon the tail reaction.

Nalorphine has a rather weak preventive effect on the tail reaction of morphine but a strong effect when given together with a large reserpine dose. Nalorphine does not eliminate the sedative action of morphine enhanced by reserpine (table 1).

DISCUSSION

Reserpine has been shown to prevent the mouse tail reaction induced by morphine (10) when very large doses of reserpine are used (10—100 mg/kg). The fact that we also obtained contrary results can be explained by the smaller doses (0.1—1 mg/kg) and by considering the action time. It has also been claimed that reserpine has no effect on the mouse tail reaction of morphine (2), but in that case the action time was extremely short. Our own results could be explained so that reserpine liberates norepinephrine more quickly than 5-HT (5) and this would be linked with the excitation phase and the enhanced tail reaction. This conforms that an adrenergic piloerection inducing DOPA enhances the tail reaction.

The sedative action of reserpine is obscure (7). We found — as others have done — that 5-HT enhances the sedative action. Because it is transferred to the brain tissue only scantily, it cannot by itself explain the sedative action of reserpine. In our experiments DOPA did not decrease the sedative action of morphine despite a simultaneous adrenergic reaction. Epinephrine is also known to produce anaesthesia (6) and therefore the possible role of the catechol amines in the sedative action is a very complicated one.

The sedative action of Hydergine, which also reversed the tail reaction enhanced by reserpine can be primary sedative or based on the adrenolytic mechanism. We prefer the latter view because Hydergine itself caused no changes. On the other hand DOPA did not antagonize reserpine in the tail reaction if Hydergine was also given.

It seems likely that reserpine and biological amines play no part in morphine-nalorphine antagonism. The changes after large doses of reserpine must be considered unspecific. Reserpine diminishes the morphine analgesia of the mouse (8, 9) and this is

considered due to the changes in the brain norepinephrine (8). However morphine causes no significant changes in the brain norepinephrine of the dog (12) although it produces deep anaesthesia that can be entirely eliminated by nalorphine.

SUMMARY

The effects of reserpine, DOPA, 5-HT and Hydergine on the action of morphine and nalorphine were studied using the mouse tail reaction of Straub-Hermann and the sedative reaction as a criterion.

1) A large dose of reserpine (1 mg/kg) prevents the mouse tail reaction induced by morphine if allowed to act for 4 hours but enhances it if allowed to act for only 1—2 hours. Small doses do not prevent the mouse tail reaction.

2) DOPA enhances the tail reaction inhibited by reserpine. This effect is eliminated by Hydergine. DOPA decreases the sedative action of reserpine but not that of morphine.

3) 5-HT increases the sedative action of morphine.

4) Hydergine reverses the tail reaction stimulating effect of reserpine.

5) These drugs do not affect the morphine-nalorphine antagonism.

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MODIFICATION OF THE ADH-METHOD IN THE DETERMINATION OF BLOOD ALCOHOL

by

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The Widmark-method (17) and the enzymatic ADH-method (4, 7, 8, 9) specific to ethanol are the most important methods for determination of the alcohol content of blood. The results obtained by these methods have been compared on the basis of extensive research material (1, 3, 5, 6, 7, 10, 12, 14, 18).

In the Widmark-method ethanol is distilled at 60°C (16). In the ADH-method the sample, deproteinized with perchloric acid, is added to a pyrophosphate buffer and, after the addition of DPN and ADH, the DPNH is measured photometrically.

Kirk, Gibor and Parker (11) presented a modification of the ADH-method in 1958 in which the ethanol was allowed to diffuse into the enzyme solution at room temperature straight from a blood sample (10 μ l) pipetted on to a piece of paper impregnated with sodium dihydrogen phosphate and magnesium chloride.

In the present work the alcohol content of blood of both living and dead subjects was determined by means of the ADH-method as modified at the beginning of 1958 (2). In this modification diffusion of the ethanol to the enzyme solution was carried out in a Widmark flask at room temperature.

Reagents

— *Buffer solution*: Sodium pyrophosphate (10 H₂O) 10 g, semicarbazide hydrochloride (Hopkins & Williams Ltd.) 5 g and glycine (Amend

Drug & Chemical Co.) 0.5 g are dissolved in ca 250 ml of water after which 10 ml of 2 N sodium hydroxide is added and the solution is diluted to 300 ml.

— *DPN solution*: Ca 1.07 mg DPN (Boehringer) in 1 ml.

— *ADH solution*: Yeast ADH (ca 40–50 mg in 1 ml buffer solution; obtainable from Biokemiska Avdelningen, Medicinska Nobelinstitutet, Stockholm, Sweden), is diluted with water to ca 7 mg ADH per ml.

— *The final solution*: Mixed by adding 2.5 parts of DPN solution and 1 part of ADH solution to 72.5 parts of buffer solution.

Procedure

Of the final solution 4 ml is pipetted into a 50 ml Widmark flask. The blood or serum and standard ethanol solutions (Merck) are weighed (100–300 mg) using Widmark capillary, blown out into a 4 ml tube with a glass stopper containing 1 or 2 ml of water, and mixed. Of these solutions 0.1 ml samples are pipetted into a cup attached to the stopper of the Widmark flask. As a reagent blank 0.1 ml of water was used. Filter paper strips of about 1×1 cm are placed in every cup. The flasks are kept at room temperature and are carefully shaken at intervals. In this investigation the measurements were made with a Beckman DU spectrophotometer at wavelengths of 340 $m\mu$ and 366 $m\mu$, in 1 cm calibrated cells. In the other details, the usual procedure of the ADH-method was followed (13, 15).

In several experiments the extinction at different intervals was investigated. The highest ethanol concentration we had in the stopper cup was 0.201% (Fig. 1).

In the experiments it became apparent that in the temperature range used (20–26°C) extinction reaches its maximum in about

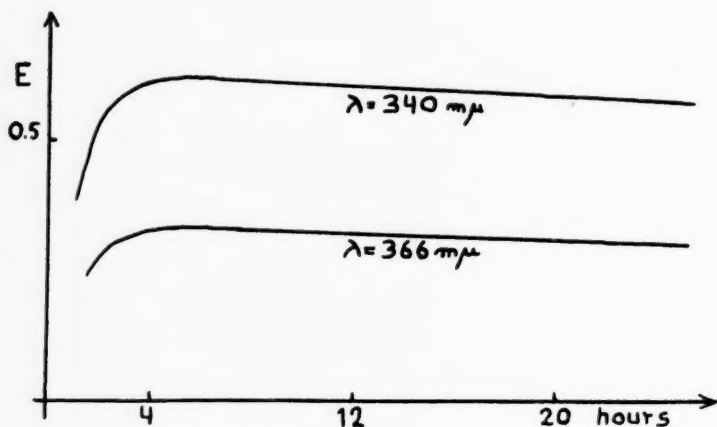


Fig. 1. The extinction as a function of time.

6 hours, after which it falls off slowly and linearly. Extinction falls at a rate of ca 0.001 per hour. In the blood alcohol determination measurements were carried out after 15—18 hours. For the ratio E_{366}/E_{340} , for which Redetzki & Johannesmeier (15), and likewise Machata (13) obtained a value of 0.565 we obtained a value of appr. 0.53. The extinctions of reagent blanks compared with water in the different test series varied between 0.050 and 0.065 at a wavelength of $\lambda = 340 \text{ m}\mu$ and between 0.020 and 0.035 at a wavelength of $\lambda = 366 \text{ m}\mu$.

In cases of drunken driving in Finland, the blood alcohol is routinely determined by the Widmark-method making three parallel analyses. In 88 cases one analysis according to the modified ADH-method presented has been taken in addition to those of the Widmark-method (W). In six cases the results of both methods were identical. The differences in the other cases (Δ) are shown in Table 1. The highest blood alcohol value obtained among these cases was $2.94^0/_{00}$.

TABLE 1

DIFFERENCES BETWEEN MODIFIED ADH-METHOD AND WIDMARK-METHOD IN DRUNKEN DRIVING CASES. NUMBER OF CASES

$\Delta^0/_{00}$	W > ADH	W < ADH
0.01—0.10	41	28
0.11—0.20	6 (+)	5 (++)
0.21—0.30	2	

(+) In 4 cases, blood alcohol (W) $> 1.5^0/_{00}$, and in two cases, (W) $> 2^0/_{00}$.
 (++) In all cases, blood alcohol (W) $> 2^0/_{00}$.

As seen from the Table 1, in 11 cases out of 88 the differences between the values obtained by the Widmark-method and the present method varied between 0.11 and $0.20^0/_{00}$, and in all of these cases the blood alcohol (W) was $> 1.5^0/_{00}$. In the two cases in which the difference (W > ADH) was between $0.21—0.30^0/_{00}$, the blood alcohol in one of the cases was $2.44^0/_{00}$. In the other case (No. 533/58 F), giving the Widmark value $1.79^0/_{00}$ and the ADH value $1.54^0/_{00}$, the person was found to be a diabetic who was taking Alentin tablets for treatment. In the medical examination of his urine the sugar reaction was positive but there were no keto substances.

The blood alcohol of 47 dead subjects was determined by both methods. Blood samples were taken 1—13 days after death and analyses carried out 1—5 days after the samples had been taken.

In 37 cases the subjects had, according to the evidence, taken liquors containing only ethanol. The highest blood alcohol value (W) was 4.67‰. The results are seen in Table 2.

TABLE 2

DIFFERENCES BETWEEN MODIFIED ADH-METHOD AND WIDMARK-METHOD IN ETHANOL DEATH CASES. NUMBER OF CASES

	Δ ‰	Number of Cases
W =, > and < ADH	0—0.10	31
W > and < ADH	0.11—0.20	4
W > ADH	0.21—0.30	

In 10 cases, according to the case histories, the deceases had taken methanol or technical alcohols containing among others methanol (denat. spirit), and in all cases methanol was found qualitatively in organ specimens taken from the deceased. The blood alcohol values and differences Δ ($\Delta = W - ADH > 0$) are shown in Table 3.

The advantage of the diffusion method is that deproteinization is avoided, and clear solutions are obtained for photometry. The diffusion method used takes more time than the ordinary ADH-method, but this has no significance in routine work.

TABLE 3

BLOOD ALCOHOL VALUES IN METHANOL AND METHANOL-ETHANOL DEATH CASES

Case No.	Widmark-value ‰	ADH-value ‰	Δ ‰
129/58	1.30	0.00	1.30
131/58	1.23	0.00	1.23
136/58	2.09	0.00	2.09
140/58	2.65	0.00	2.65
158/58	0.80	0.20	0.60
174/58	0.49	0.00	0.49
198/58	0.81	0.01	0.80
738/58	1.73	0.86	0.87
32/59	0.88	0.10	0.78
778/59	1.01	0.00	1.01

SUMMARY

The paper presents a modification of the ADH-method in which the diffusion of ethanol in enzyme solution has been carried out in a Widmark flask at room temperature. This method has been used in addition to the Widmark-method for determination of the alcohol content of blood in 88 drunken driving cases and in 47 death cases of alcohol poisoning, ten of whom had died of methanol or mixed methanol/ethanol poisoning.

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VARIATIONS IN THE ASH CONTENTS OF BONES OF THE EXTREMITIES¹

by

PEKKA VIRTAMA

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Interest in investigation of mineral content of bones has recently greatly been increased owing to several new radiological methods for evaluation of «bone density» (4, 5, 6, 9, 14). There are many pitfalls in the methods used mainly due to non-mineralized tissue inside and surrounding the bones to be investigated, as pointed out by Omnell (8). However, under certain conditions the blackening of X-ray images of bones surrounded by a relatively thin layer of non-mineralized tissue is related to their actual mineral contents (7, 11, 12, 13).

Radiological methods have been used in follow-up studies in analysing changes of bone density due to some local pathologic condition (8), and in comparison of mineralization grades of given groups of population (10). However, the most important problem from the clinical point of view is estimation of the mineralization grade of a single individual. Even if there were a dependable radiological method for determination of the true mineral content of bones, it hardly would be possible to examine all bones of the skeleton. Baker and Schraer (1) examined this problem and found that the «total dry skeletal weight» can be predicted with a reasonable accuracy by means of a densitometric evaluation of X-ray images of the humerus and the femur.

The distal bones of the extremities are best accessible for

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radiological methods for bone density estimation. Therefore, mineral contents of these bones were examined and compared with each other in this work.

MATERIAL AND METHODS

The series examined consisted of eight dissected subjects from the Department of Anatomy, University of Helsinki. Eighteen bones were removed from each one: the phalanges of the thumb and the little finger, the first and the fifth metacarpal bones, the corresponding bones of the feet, the scaphoid, the semilunar, the astragalus and the calcaneum. Thus, altogether 288 bones were examined. They were cleaned mechanically, their volumes were measured pycnometrically and their ash contents determined by burning them at 1000°C for five hours and by weighing the ash with a precision balance. The ash contents were expressed as g cm^{-3} .

RESULTS

Mean ash content of all the bones examined was 0.418 g cm^{-3} , as seen in Table 1. The mean ash content of bones of the right side

TABLE 1

Case No.	Mean Ash Content, g cm^{-3}	
	Left Side	Right Side
1	0.392	0.465
2	0.414	0.423
3	0.449	0.506
4	0.415	0.437
5	0.491	0.535
6	0.413	0.425
7	0.291	0.340
8	0.328	0.354
Mean	0.400	0.436

was about 9 per cent higher than that of the left side, as seen in Table 2.

The left-right difference of bone density was further analysed with the aid of standard statistical methods (3). The following regression equation was obtained:

TABLE 2
DIFFERENCES OF ASH CONTENTS OF BONES OF THE LEFT AND THE RIGHT SIDE,
PER CENT

Case No.	1	2	3	4	5	6	7	8	Mean
Bone:									
Scaphoid	12.3%	-1.4	24.8	1.7	11.0	19.9	2.7	2.7	9.6
Semilunar	12.1	-1.6	24.1	—	11.5	30.9	2.3	2.9	12.2
1st metacarpal ..	40.0	-2.9	14.0	6.5	8.2	15.0	2.7	-0.7	11.2
5th " ..	15.7	2.8	12.6	6.3	10.8	14.6	10.8	9.2	10.3
1st ph., 1st finger	20.2	14.3	6.1	13.2	12.4	13.0	17.2	3.5	12.5
2nd " "	19.6	6.5	11.2	12.5	12.2	7.0	8.1	6.9	10.5
1st " 5th "	28.2	14.7	8.2	7.4	10.5	5.7	10.7	0.8	10.8
2nd " "	26.2	14.3	8.5	6.4	10.7	6.9	8.7	1.0	10.3
3rd " "	24.9	12.7	7.1	6.2	11.8	4.0	9.3	1.8	9.7
calcaneum	5.8	3.0	4.8	7.7	-1.3	21.0	11.4	27.6	10.3
astragalus	4.9	4.7	—	2.8	-1.1	5.4	9.0	12.6	5.8
1st metatarsal ..	15.7	-7.1	18.9	8.8	9.2	13.7	10.7	1.6	10.7
5th " ..	2.5	0.5	-1.2	6.6	9.6	9.9	10.2	2.0	5.4
1st ph., 1st toe ..	10.4	3.2	14.4	14.8	22.5	8.8	6.3	6.8	10.9
2nd " " ..	17.0	2.5	31.2	12.6	19.0	6.7	11.5	7.0	13.5
1st " 5th " ..	14.4	16.2	30.1	-6.9	-11.2	25.1	1.9	2.4	17.8
2nd " " ..	13.4	-8.7	28.4	-10.9	-5.2	26.3	-0.9	1.7	11.9
3rd " " ..	9.1	-7.6	17.8	-7.2	1.8	15.8	2.5	1.4	7.9
MEAN	15.7	2.1	17.6	5.3	8.2	14.4	7.9	2.8	9.3

Negative number means that ash content of the left bone was higher.

$$Y = 1.2X - 0.036 \text{ (g cm}^{-3}\text{)},$$

where Y is the ash content (g cm⁻³) of bones of the right side, X that of the left side. The left-right difference was also analysed by means of t-test and found to be highly significant ($P < 0.001$).

The difference in the ash contents of bones of the left and the right side was most marked in subjects which had been manual workers before their deaths. Case No. 1 (Table 2) was a shop-assistant, 58 years of age, No. 3 was a farmer 51 years of age, and 6 a 53 years old carpenter. On the other hand, No. 2 was a 78 years old pensioned worker, No. 4 a 58 years old businessman, 7 a former dairymaid, 62 years of age, and 8 a 81 years old mental patient. The occupation in case No. 5 was unknown. Marked osteoporosis was seen in cases No. 7 and 8, which also had lowest ash contents of the bones examined. In these cases the left-right differ-

ence was quite slight. Thus it is apparent that old subjects who had used their extremities only in most necessary functions had the most even bone density.

Ash content of bones of the same extremity and even of the same finger was variable. Therefore, relations of some bones were analysed statistically as seen in Table 3. Regression was highly significant in every case examined ($P < 0.001$). This fact does not imply that one of the bones examined could be used as an indicator of mineral contents of all other bones examined. In order to illuminate this problem, the original standard deviations were compared with the residual standard deviations left after the regression equations had been fitted (2). As can be seen in Table 3, 57–86 per cent of the original variation was explained by the regression, which is not too bad.

Ash content was best related in similar bones. Thus, small long bones with small marrow cavities showed higher ash content than long bones with big marrow cavities or cancellous bones. Therefore, correlation between the volume and the ash content (g cm^{-3}) was examined statistically. The regression was not significant, however. Apparently the volume is dependent on growth factors and does not change according to functional requirements after having reached a given stage.

The individual variation of ash contents of various bones is more difficult to explain with functional differences than the left-right variation. However, in some cases there was an apparent cause for individual differences. *E.g.* in case N:o 3 the subject had drum-stick fingers probably due to chronic pulmonary tuberculosis and emphysema. The ash content of the relatively big distal finger phalanges was unusually low in this case.

CONCLUSIONS

The good correlation obtained between ash contents of various bones of the extremities gives reason to assume that it would be possible to estimate the mineralization grade of a given group of population by measuring the mineralization grade of one bone only. However, in estimating the mineralization grade in a single case considerable care is necessary. Bones of the extremities, particularly those of the hands, are easily exposed to local traumata

TABLE 3
REGRESSIONS OF ASH CONTENTS OF SOME BONES EXAMINED

Bones Compared:		Regression Equation	Correlation Coefficient	Variation Explained by Regression
X	Y			
1st phalanx, 1st finger	2nd ph., 1st finger	$Y = 0.92X + 0.066$	0.91	86 per cent
"	1st metacarpal	$Y = 1.01X - 0.036$	0.98	85 " "
"	1st ph., 5th finger	$Y = 1.10X + 0.008$	0.92	65 " "
"	scaphoid	$Y = 0.78X + 0.057$	0.91	60 " "
"	calcaneum	$Y = 0.58X + 0.088$	0.80	57 " "
"	1st metatarsal	$Y = 0.91X - 0.011$	0.81	63 " "
"	1st ph., 1st toe	$Y = 1.05X - 0.010$	0.83	85 " "
"	1st ph., 5th toe	$Y = 1.09X - 0.039$	0.92	75 " "
1st phalanx, 5th finger	2nd ph., 5th finger	$Y = 1.17X - 0.071$	0.96	79 " "
1st metacarpal	5th metacarpal	$Y = 0.96X + 0.004$	0.98	85 " "

and strains. Furthermore, osteoporotic processes affecting various bones of the skeleton may be very different. Thus, it is hazardous to predict the mineralization grade of an extremity or the whole skeleton based on radiological measurements of one bone only.

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URINARY EXCRETION OF THIAMINE, RIBOFLAVIN,
NICOTINIC ACID, PANTOTHENIC ACID AND BIOTINE
IN ANEMIC AND NON-ANEMIC CARRIERS OF THE FISH
TAPEWORM (*DIPHYLLOBOTHRIUM LATUM*)

by

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Using microbiological methods, Nyberg (7) established that water extract of the fish tapeworm (*Diphyllobothrium latum*) contains an abundance of vitamin B₁₂; the average vitamin B₁₂ activity was 2.3 µg of B₁₂ per gm of dry worm substance. The corresponding value for *Taenia saginata* was 0.046 µg and for *Ascaris lumbricoides* 1.8 µg of B₁₂. In his paper chromatographic analyses, he found, also, that the same water extract of the fish tapeworm contained desoxyribosides of guanine, cytosine and thymine. In his later communication, Nyberg (8) studied the uptake and distribution of Co⁶⁰ — labelled vitamin B₁₂ by the fish tapeworm. About 44% of a single oral dose of radioactive vitamin B₁₂ given to the host was absorbed by the worm. The radioactivity was mainly concentrated in the proximal part of the worm.

Nyberg's results support the well-known theory, presented by Bonsdorff (1, 2, 3), that the host and the parasite compete for vitamin B₁₂. A deficiency of B₁₂ may result and cause pernicious anemia in the host, especially if the tapeworm is situated in the proximal part of the small intestine.

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Most reports concerning the correlation between vitamins and fish tapeworm, or fish tapeworm anemia, are concentrated on the vitamin B₁₂. It is not quite unlogical, we believe, to assume that the fish tapeworm, living in the intestinal canal, might disturb also the nutritional state of other vitamins in the host. In this study we have followed the nutritional state of thiamine, riboflavin, nicotinic acid, pantothenic acid, and biotine in the anemic and non-anemic carriers of fish tapeworm. We have not been able to find reports of similar investigations in the literature. Vartio (13) studied the cocarboxylase content of blood in normal subjects and in various diseases. His material includes 5 patients with helminthiasis. Their average cocarboxylase content in blood was 13.9 γ /100 ml; the corresponding value for the normal subject was 14.9 γ /100 ml of blood. A worm cure with filicin had no effect on the content of cocarboxylase in blood of the carriers of fish tapeworm. The blood values of these patients are not given.

MATERIAL AND METHODS

Our series comprised 28 patients with fish tapeworm. Eight of these had fish tapeworm anemia, and 20 carriers were non-anemic. Vitamin determinations on worm substance were done on two occasions, and the results were compared with those obtained on fresh calf liver. Our control group consists of 7 healthy persons hospitalized for mild subjective symptoms without objective findings.

The 24-hour output of urine was collected from each patient on three successive days into vessels protected from light. The urine was measured and samples put into test tubes; a few drops of 10% hydrochloric acid was then added. The test tubes were protected from daylight and kept in a refrigerator at a temperature under +5°C. The thiamine, riboflavin, nicotinic acid, pantothenic acid, and biotine were microbiologically analysed as follows:

Thiamine: Before determination, the urine samples were treated with 2.5 times the amount of N/2 H₂SO₄ for a period of 10 minutes in steam at 120°C, titrated to pH 6.6 and finally diluted to a twentieth part. Ten determinations were made on each sample so that two readings from each of four concentration levels were obtained, and, in addition, determinations were made on two urine samples, to both of which a known quantity of the vitamins to be studied was added. If on determination at least six readings differing by at the most $\pm 10\%$ from one another were not obtained, then the whole analysis was repeated. For the test, the organism *Lactobacillus fermentum* 36 ATCC 9338 was used and the determinations were made according to Sarett and Cheldelin (9). The turbidity caused by the

bacterial growth was read by Hilger's photometry method. From the urine samples taken on three successive days, the mean of the vitamin content was assessed, each mean thus being based on at least 30 analyses. The same principles were observed in the following tests as well.

Riboflavin: The samples were treated according to the same procedure of extraction except that the samples were kept for 15 minutes under a 15 lb pressure at 120°C. The samples were diluted to one-fiftieth part and analysed according to the same principles as in the previous group, but the test strain used was *Lactobacillus casei* ATCC 7469 (9).

Nicotinic acid and nicotinamide: Dilution and mode of extraction of the samples were the same as for determination of riboflavin. Snell and Wright's (12) method, and the test strain *Lactobacillus arabinosus* 17-5 ATCC 8014 was used. The results of the analysis was read by turbidimetry, as in the preceding group.

Pantothenic acid: The urine samples were diluted and added straight into the determination tubes. The analysis was done according to Skeggs and Wright (10). The test organism used was *Bacillus arabinosus* ATCC 8014. The results were read by turbidimetry.

Biotine: The determinations were made according to Luckey *et al.* (6), *Lactobacillus arabinosus* 17-5 ATCC 8014 being used as the test organism. The urine samples were diluted and added to the determination tubes without preceding extraction. After sterilization, inoculation, and 18 hours' incubation the turbidimetric readings were made.

RESULTS

Most of the results obtained are presented in Tab. 1. In the patients with fish tapeworm anemia, the decreased excretion of thiamine was highly significant as compared with the healthy subjects ($T = 5.36$; $p < 0.001$) and with the non-anemic carriers of fish tapeworm ($T = 4.43$; $p < 0.001$). In the non-anemic carriers of fish tapeworm, excretion of thiamine was also significantly lower than in the healthy subjects ($T = 3.41$; $p < 0.01$). The riboflavin and nicotinic acid excretion means did not differ significantly in the patients with fish tapeworm anemia, in the non-anemic carriers of fish tapeworm, and in the healthy controls. The excretion of pantothenic acid was significantly lower in the patients with fish tapeworm anemia than in the non-anemic carriers of fish tapeworm ($T = 3.29$; $p < 0.01$) and in the healthy subjects ($T = 3.49$; $p < 0.01$). The non-anemic carriers of fish tapeworm did not differ significantly from the healthy subjects in this respect ($T = 1.9$). The average excretion of biotine did not differ significantly in the groups studied (the T -value for the difference between the patients with fish tapeworm anemia and the healthy subjects is 1.6).

TABLE 1

URINARY EXCRETION OF THIAMINE, RIBOFLAVIN, NICOTINIC ACID, PANTOTHENIC ACID AND BIOTINE IN PATIENTS WITH FISH TAPEWORM ANEMIA, IN NON-ANEMIC CARRIERS OF FISH TAPEWORM, AND IN HEALTHY SUBJECTS

Group	Subject	Sex	Age Years	Hgb, g/100 ml	R.B.C. mill. per c. mm.	Gastric Acid Secret. + or —	Urinary Excretion of Vitamins Expressed in $\mu\text{g}/24$ Hours				
							Thia- mine	Ribo- flavin	Nico- tinic Acid	Panto- thenic Acid	Bio- tine
<i>Patients with fish tapeworm anemia:</i>	1	M	26	6.4	1.80	—	65	661	735	2853	2.73
	2	F	47	5.8	1.70	—	39	740	760	3801	8.60
	3	M	53	4.9	1.40	—	36	269	1329	1829	12.40
	4	F	22	9.0	3.60	+	65	348	1036	1268	5.70
	5	F	74	6.3	1.50	not ex.	27	510	716	1335	4.70
	6	F	60	10.2	2.21	—	44	276	2617	2245	7.90
	7	M	60	9.8	2.30	—	99	1814	2948	4428	25.20
	8	F	26	9.7	2.20	+	91	999	1185	3547	8.20
Mean			46	7.8			58	702	1420	2413	9.47
<i>Non- anemic carriers of fish tape- worm:</i>	9	F	74	12.0	— ?	+	140	961	863	1477	9.10
	10	F	66	12.5	4.40	+	101	301	1027	2032	6.80
	11	F	22	13.9	4.31	+	148	375	847	3122	8.30
	12	M	63	13.0	4.16	+	132	750	1763	3004	19.70
	13	M	51	14.7	4.40	+	51	1367	1774	4734	14.40
	14	F	43	13.3	4.26	+	69	102	1200	2700	10.30
	15	M	43	14.1	4.78	+	102	938	1530	5043	12.70
	16	M	33	13.9	4.53	+	103	1627	2251	2816	11.20
	17	M	40	16.3	4.64	+	100	1182	2688	6107	16.20
	18	F	24	13.9	4.54	+	146	1328	2379	6506	8.60
	19	M	69	17.7	4.49	+	196	838	1925	2104	7.40
	20	F	23	12.9	4.11	+	123	544	1417	4897	6.70
	21	M	63	13.5	—	+	331	489	1278	5097	8.30
	22	F	41	14.3	4.38	+	199	1339	1396	4925	12.40
	23	M	58	16.7	5.55	+	456	1824	1326	7316	16.40
	24	F	35	12.8	3.60	+	106	1053	1287	3915	10.60
	25	M	64	14.3	5.08	+	188	679	931	2721	6.60
	26	F	23	13.1	4.18	+	121	871	1357	4183	7.70
	27	M	58	14.3	4.04	—	100	868	1434	4450	12.80
	28	M	39	14.5	5.30	+	191	794	1458	4123	19.10
Mean			46.6	14.1			155	912	1507	4061	11.30
<i>Healthy subjects:</i>	29	M	44	12.0		+	205	707	1272	2589	3.90
	30	F	50	11.4		+	253	473	1907	5743	24.20
	31	M	41	16.0		+	141	1523	2189	7589	15.80
	32	M	27	11.9		+	500	1676	1776	6202	39.50
	33	M	36	13.8		+	438	1713	1264	4220	15.00
	34	M	56	15.9		+	327	518	1438	10379	9.80
	45	M	57	16.8		+	610	1214	1586	4246	16.20
Mean			44	14.0			355	1121	1633	5853	17.70

In one patient with fish tapeworm anemia (Case No. 3) the excretion studies were performed one month after the worm cure without the patient having been given other treatment.

The results were as follows:

	Hgb	MCH	Thiamine	Ribo- flavin	Nicotinic acid	Panto- thenic acid	Bio- tine
Before worm cure	4.9	36	36	269	1,363	1,829	12.41
One month later	10.8	34	194	968	1,616	2,414	20.60

All excretion values had increased and the fish tapeworm anemia had been almost cured.

In two instances the vitamin determinations were performed on the expelled worm. The carriers of these worms were non-anemic. After washing the worm, it was dried between filter paper, weighed, and the results of the analyses expressed in micrograms (biotine in millimicrograms) per wet weight gram. Simultaneously, vitamin determinations on fresh calf liver were performed with the same method.

The results were as follows:

	Thiamine	Ribo- flavin	Nicotinic acid	Panto- thenic acid	Biotine
Fish tapeworm No. 1	4.4	2.75	52	3.4	56.87
" " No. 2	1.6	1.95	24	0.77	16.04
Fresh calf liver	2.0	6.30	59	lacking	lacking

As is seen, the thiamine, riboflavin and nicotinic acid content in fish tapeworm and fresh calf liver are of the same magnitude.

DISCUSSION

On the basis of our results it may be considered true that the excretion of thiamine is reduced in patients with fish tapeworm anemia, and probably also in the non-anemic carriers of fish tapeworm. Excretion of pantothenic acid is also decreased in the patients with fish tapeworm anemia in comparison with healthy subjects; in non-anemic carriers of fish tapeworm this is no more distinctly observed. The vitamin excretion after tapeworm expulsion seems to be increased, to judge from one patient studied. Vitamin determinations on tapeworm showed that it contained an abundance

of the vitamins, thiamine, riboflavin and nicotinic acid, as did the fresh calf liver.

Our results seem to show that the capacity of the fish tapeworm to cause a deficiency in B₁₂ vitamins, as is known from the literature, is not a temporary phenomenon, and may concern other vitamins as well — according to our results, at least thiamine and pantothenic acid. It is true that we cannot draw any definite conclusions on the basis of the decreased excretion of urine, that is, whether there is a question of a deficiency condition or not. The fact that in the patients with fish tapeworm anemia, particularly the excretion value for thiamine and pantothenic acid were lower, may partly be due to at least four of these patients having achlorhydria in which in any case the thiamine and riboflavin excretion is known to be distinctly reduced, even without the presence of tapeworm (4). However, the excretion values were markedly reduced also in the patients with fish tapeworm anemia whose gastric acid secretion was normal. Our material does not permit us to evaluate to what extent the symptoms frequently appearing in fish tapeworm carriers — giddiness, aural tinnitus, nausea, etc. — may be associated with the reduced vitamin excretion values observed. Nor can we say whether the reduced thiamine excretion noted and also the decreased pantothenic acid excretion in patients with fish tapeworm anemia is due to the worm utilizing or destroying the vitamins mentioned and thus preventing the host from obtaining sufficient amounts for its own use, or, whether the worm, so closely adhering to the mucous membrane of the intestine, absorbs these vitamins from the host organism for its own use. On the grounds of studies made on the rat tapeworm (*Hymenolepis diminuta*), Chandler *et al.* (5) hold that tapeworms have the capacity to absorb various vitamins B and that this capacity varies in the different species of tapeworm.

SUMMARY

1) Urinary excretion of thiamine, riboflavin, nicotinic acid, pantothenic acid, and biotine has been studied in 8 patients with fish tapeworm anemia, in 20 non-anemic carriers of fish tapeworm, and in 7 healthy subjects.

2) Urinary excretion of thiamine and pantothenic acid was significantly lower in the patients with fish tapeworm anemia than in the non-anemic carriers of this worm and in the healthy subjects.

3) Urinary excretion of thiamine was significantly lower in the non-anemic carriers of fish tapeworm than in the healthy controls.

4) Urinary excretion of riboflavin, nicotinic acid and biotine did not differ significantly in the groups studied.

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EFFECT OF ORAL NEOMYCINE TREATMENT ON BLOOD LIPIDS AND INTESTINAL ABSORPTION OF VITAMIN A

by

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Recently, it has been reported that neomycine sulphate, administered orally, has a decreasing effect on the cholesterol level of serum in man (19, 20). The mechanism of this phenomenon is as yet undiscovered.

In addition to the action of neomycine sulphate given by mouth on the cholesterol level — we have made an endeavour in the present study, to clarify its effect on other lipids and on the resorption of fat from the intestine, using the vitamin A absorption test as an index of the resorption of fat from the intestine (3).

MATERIAL AND METHODS

Our series comprised a total of 33 patients (aged 30—60) treated in hospital for coronary disease. Before starting neomycine treatment, the patients were observed for three days at the least in the hospital. The patients were divided into two groups as follows:

Group 1: 16 patients, these were given 1.65—2.0 g of neomycine sulphate orally for 9—27 days. In all cases, the serum total cholesterol was noted before starting, and during the treatment. In 10 cases of this group, determinations of other lipids were also made before treatment and for 6—12 days during the treatment.

Group 2: 17 patients; the same amount of neomycine was given daily for four days to these patients as in Group 1. The vitamin A absorption test was performed before starting treatment and on the fourth day of treatment. Total plasma cholesterol determinations were made on the same days.

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Analysis of Serum Lipids. — Total cholesterol was determined by the p-toluenesulphonic acid method (13).

Fractionation of serum lipids in 3 fractions was performed by silicic acid chromatography (5) with some modifications (6). The following fractions were determined:

Sterol esters and free cholesterol, by the Tshugaeff-reaction (7).

Glycerides, by the hydroxamate reaction (21).

Lipid phosphorus, by the Fiske-Subbarow method.

For a more detailed description of these methods we refer the reader to Haahti and Nikkilä's (6) work.

The vitamin A absorption test was performed as follows: A dose of 300–350,000 International Units of vitamin A was administered as a solution in oil (Arovit, Hoffman La-Roche). Blood samples (heparin as anticoagulant) were taken initially and after 4 hours. The patients were not kept fasting during the test. The rise of the plasma vitamin A level during a 4 hours' test period is the measure of A vitamin absorption.

Vitamin A was estimated by the spectrophotometric method involving the destruction of vitamin A by ultraviolet irradiation (18, 17). Heparinized plasma (1.5 ml) was extracted with 3.0 ml absolute alcohol and 3.0 ml n-heptane (E. Merck AG, Darmstadt). The optical density of 2.5 ml of heptane layer was determined before and after irradiation, using non-irradiated or irradiated n-heptane for the blank setting, in a Beckman DU quartz spectrophotometer at a wave-length of 327 m μ . Heptane specimens were irradiated for 2½ hours at a distance of 4 cm from a Hanau ultraviolet ray lamp (Stab-Analysenlampe S 81, Quartzlampen Ges. MBH, Hanau). The reading of the test is the difference between optical densities of non-irradiated and irradiated samples. The rise of the vitamin A plasma level was calculated, using the $E_{1\%}^{1\text{cm}}$ value 1750 for A-vitamin alcohol (WHO's Expert Committee on Biological Standardization).

RESULTS

Table 1 illustrates the effect of oral neomycine on the total cholesterol of serum. In 11 of the 16 patients, a decrease in the cholesterol level was noted. Patients with high initial serum cholesterol showed a more marked decrease during neomycine administration. The mean decrease is not statistically significant.

Table 2 represents the effect of neomycine on the different lipid components. The ratio between free and esterified cholesterol remained the same (about 0.4) during the treatment. The difference in the mean for sterol esters before neomycine treatment and during it is statistically significant ($T = 2.93$, $p < 0.01$). In 7 of the 10 subjects in this series, a decrease in glycerides was observed.

TABLE 1

EFFECT OF ORAL NEOMYCINE SULPHATE ON THE TOTAL CHOLESTEROL CONTENT OF BLOOD; DETERMINATIONS PERFORMED EACH 3RD DAY

Period	Without Neomycine		Neomycine Sulphate, 1.65—2.0 g/day, oral								Change
Day	1	3	6	9	12	15	18	21	24	27	
Subject											
1	265	275	240	265	270	300					— → +
2	320	315	285	245	240	245	250	250			—
3	305	285	305	260	270	220					—
4	322	290	295	302	290	290	290				0
5	370	350	310	310	280	300	270	280			—
6	975	970	865	730	725	725					—
7	295	305	285	240	235	270	280				—
8	365	340	295	270	260	295	290	290	260	270	—
9	375	355	330	300	275	255	250	280	285		—
10	210	215	210	185	180	200					—
11	310	285	250	270	270	245	275				— → +
12	270	285	285	255	225	245					—
13	280	285	270	260	245	250	260				—
14	485	445	415	335	320	290					—
15	335	330	320	325							0
16	405	405	360	315	325	395	410	395			— → +
Mean	368	358	333	304	294	302	286	299	272		
Standard deviation	168	166			120		46				
Standard error	42	42			31		15				

The means do not differ from one another. The phospholipids decreased during the treatment in nine cases, and the mean value is probably significant ($T = 2.80$, $p < 0.05$).

The effect of neomycine on the vitamin A absorption is presented in Table 3. In 16 of 17 patients, decreased values were noted in the vitamin A absorption test during neomycine treatment. Figure 1 shows that the more the plasma vitamin A level rises, as noted before treatment, after oral administration of vitamin A, the greater is the fall in this increase during neomycine treatment. If we consider that, in the vitamin A absorption test, a normal increase is at least 500 I.U. in the plasma vitamin A level (King and Wootton 1956), then a pathologically low increase during

TABLE 2

EFFECT OF NEOMYCINE TREATMENT ON THE BLOOD LIPIDS DETERMINED BY THE SILIC ACID CHROMATOGRAPHY METHOD

Subject	Before Neomycine Treatment					On 6th--12th Day during Neomycine Treatment				
	Sterol Esters I mg %	Free Cholesterol II mg %	I + II mg %	Neutral Fat μ M/ml	Phospho-Lipids mg %	Sterol Esters I mg %	Free Cholesterol II mg %	I + II mg %	Neutral Fat μ M/ml	Phospho-Lipids mg %
1	166	70	236	7.8	7.8	145	69	214	10.9	8.6
2	215	86	301	10.4	11.5	162	56	216	7.1	7.1
3	235	71	306	15.5	13.2	154	50	204	7.1	7.7
4	122	50	172	4.8	7.8	99	46	145	4.0	4.0
5	170	72	242	4.0	8.9	152	63	215	4.0	7.3
6	164	60	224	6.2	9.1	130	55	185	5.4	7.9
7	174	68	242	6.3	9.3	154	55	209	5.5	7.0
8	285	134	419	6.5	14.3	168	76	244	4.5	8.2
9	220	95	315	13.6	11.8	220	85	305	7.5	10.2
10	240	150	390	34.0	16.5	164	158	322	54.0	13.9
Mean	199	86	285	10.9	11.0	155	71	226	11.0	8.2
Standard deviation	45.3	30.7			78.3	29.0	31.1			1.8
Standard error of the mean	14.1	9.6			0.83	5.4	9.7			0.56

neomycine treatment is observed in 15 of 17 cases. Table 3 shows as well that the changes occurring simultaneously in the plasma total cholesterol are not correlated with the results obtained in the vitamin A absorption test.

DISCUSSION

To judge from our results, it seems evident that a decrease in the blood total cholesterol occurs at least in the beginning of neomycine treatment. If the series presented in Tables 1 and 3 are combined it is seen that, during the first week of treatment, there occurred a decrease in the cholesterol level in 27 of 33 patients. The decrease is not, however, quite evident. The misleading effect of hospitalization (change in diet) on the results presented may be excluded (9).

TABLE 3

EFFECT OF FOUR-DAY ORAL NEOMYCINE TREATMENT ON THE INTESTINAL ABSORPTION OF VITAMIN A AND ON THE CHOLESTEROL LEVEL OF THE PLASMA

Subject	Vitamin A Absorption Test. I.U. of Vitamin A per 100 ml of Plasma			Plasma Cholesterol Level mg/100 ml of Plasma		
	Before Neomycine Treatment	On 4th Day During Neomycine Treatment	Difference	Before Neomycine Treatment	On 4th Day During Neomycine Treatment	Difference
1	630	410	— 220	445	340	— 105
2	3500	100	— 3400	370	285	— 85
3	280	230	— 50	250	230	— 20
4	580	300	— 280	305	265	— 40
5	510	240	— 270	—	—	—
6	1300	840	— 460	260	270	+ 10
7	860	190	— 670	295	255	— 40
8	360	34	— 326	210	200	— 10
9	2300	340	— 1960	355	280	— 75
10	280	65	— 215	305	325	+ 20
11	1800	0	— 1800	330	320	— 10
12	660	140	— 520	350	275	— 75
13	1390	260	— 1130	440	405	— 35
14	320	370	+ 50	370	345	— 25
15	1310	278	— 1032	330	330	0
16	1240	125	— 1115	320	320	0
17	890	880	— 10	225	210	— 15

A more remarkable effect of neomycine is seen in the vitamin A absorption test, in which the results noted during neomycine treatment were almost always pathological. To some extent the result is surprising as it is known from tests on animals that at least penicillin, oxytetracycline and chlortetracycline sooner increase the absorption of vitamin A (2, 4, 11, 15, 16). However, Hartsook *et al.* (8) considered that chlortetracycline did not increase the utilization of vitamin A in the male albino rat, as judged from liver and kidney storage, growth, or time of survival. Neomycine causes mild diarrhea in many patients. It might thus be thought that the increased motility of the intestine might be the cause of the weakened resorption of vitamin A from the intestine. It is known, that, for instance, in enteritic conditions, slightly decreased plasma vitamin A levels may be recorded (14). On the other hand, in at least half of our patients, no marked objectively noted diarrhea developed

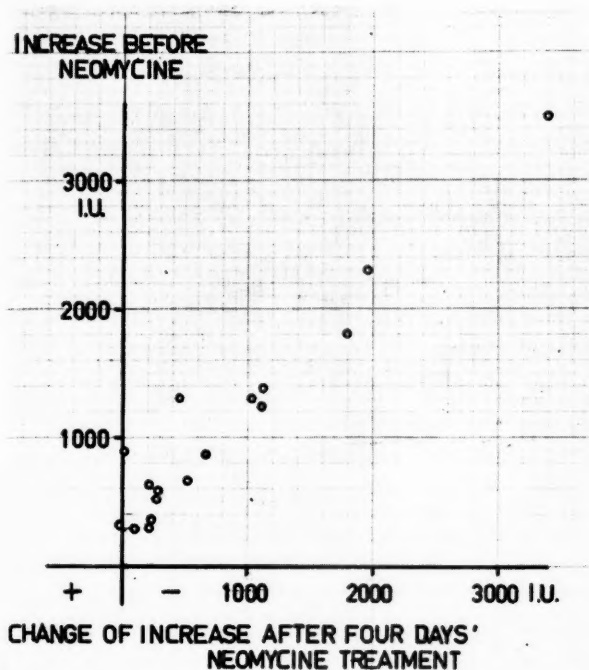


Fig. 1. — Correlation between the initial increase in the vitamin A content of plasma in the vitamin A absorption test and the change of this increase after four days' neomycine treatment.

and, in addition, it seems unlikely that the correlations seen in Figure 1 would have arisen if the cause of the weakening of the vitamin A absorption would have been the increased motility of the intestine alone.

Ninety-seven per cent of the orally administered neomycine appears non-absorbed in the faeces. Samuel (19) administered neomycine also intramuscularly and no decrease in the cholesterol level was noted. Considering this observation, it seems probable that the effect of neomycine on the vitamin A absorption is due either to changes in the intestinal flora or to some local effect on the intestinal mucosa. However, also the direct or indirect effect of neomycine on other factors in fat absorption must be paid attention to. When phthalylsulphathiazol, isoniazid, dihydrostreptomycin, oxytetracycline, polymyxin B sulphate, bacitracin

and novobiocin were given orally, no appreciable changes in the serum cholesterol content resulted (19). This observation might favour the assumption that the cholesterol level is specially associated with neomycine. As many of the above mentioned substances have even quite a broad antibacterial spectrum, it seems as if this observation would, to some extent, speak against the effect being founded directly on the intestine being sterilized by neomycine.

An interesting circumstance noted in our results is that, with regard to both the cholesterol level and the vitamin A absorption test, the greatest change occurred when the cholesterol level was initially high, or a high pre-treatment rise was obtained in the results of the vitamin A resorption test. We have previously observed the same phenomenon in connection with sitosterol (10). The higher the initial serum cholesterol values, the greater was the decrease obtained during sitosterol treatment. Beaumont and Lengère (1) have observed that in half of their patients with coronary atherosclerosis, curves, which were above the normal were obtained in the vitamin A absorption test. It seems evident that at least neomycine and sitosterol, which act mainly inside the intestine, have a «levelling» effect on this type of increased absorption of lipids. The mechanism active as regards neomycine is still unknown. Neomycine as a cholesterol-lowering agent seems to us of limited importance in practice.

SUMMARY

1) The writers have studied the effect of oral neomycine therapy on serum total cholesterol in 33 patients; in 27 of these there occurred a decrease in the serum cholesterol level at least in the first week. In some cases there was a return to the pre-treatment level during the treatment.

2) The sterol esters and the free cholesterol decreased uniformly during the treatment; also the phospholipids decreased; the changes in the neutral fat differed to some extent.

3) In 17 patients, the vitamin A absorption test was performed on the fourth day during neomycine treatment. In 16 patients, a marked weakening in the absorption of vitamin A occurred during the treatment. In 15 patients, the results must be considered

pathologically low in this connection. The change was greater, the better the response to the oral vitamin A administration obtained in the pre-treatment phase.

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PYRUVIC ACID IN THE BLOOD AND SYNOVIAL FLUID OF PATIENTS WITH RHEUMATOID ARTHRITIS

by

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We have previously studied the urinary excretion of thiamine, riboflavin, nicotinic acid, and pantothenic acid in patients with rheumatoid arthritis (6). In the present work we have studied the pyruvic acid concentration in blood and synovial fluid of patients with rheumatoid arthritis.

MATERIAL AND METHODS

Our series consist of 55 patients with rheumatoid arthritis (15 men, 40 women, mean-age 50.5 years). The normal control group includes 20 healthy students (10 men, 10 women). Groups of patients with pneumonia (12 cases), pleuritis (9 cases), malignant tumours (9 cases), and with thyrotoxicosis (22 cases) act as «pathological» controls.

The pyruvic acid (PA) content of synovial fluid was determined in 22 patients with rheumatoid arthritis. The joint aspirated was always the knee. In 5 cases the synovial fluid was aspirated before and after the intra-articular injection of hydrocortisone acetate (62.5 mg), and subjected to analysis of PA.

All specimens for PA determination were taken in the morning after an overnight fast, and with minimal bodily exercise before the

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taking of the specimens. The determination of PA was performed according to the method presented by Bonting (1) with the exception that the volumes used in the study were in millilitres, and the blood and synovial fluid samples were deproteinized by precipitation with trichloroacetic acid.

RESULTS

Controls. — The average PA concentration in blood of 20 healthy students was 0.83 ± 0.041 mg% (SD 0.184 and range 0.53—1.29).

The average PA concentration in the blood of 12 patients with *pneumonia* was 1.22 mg% (range 0.92—1.70), in 9 patients with *pleuritis* 1.19 mg% (range 0.66—1.79), in 9 patients with *malignant tumours* 1.43 mg% (range 0.93—1.76), and in 22 patients with *thyrotoxicosis* 1.20 mg% (range 0.59—2.14).

Rheumatoid arthritis: The average blood level of PA in the patients with rheumatoid arthritis was 1.15 ± 0.047 mg% (SD 0.35 and range 0.60—2.35). The difference between the rheumatoid group and the healthy students is highly significant ($T = 5.16$; $p < 0.001$).

The length of history of rheumatoid arthritis was < 5 years in 29 patients; the average blood level of PA in this group was 1.19 mg%. Rheumatoid arthritis had lasted ≥ 5 years in 26 patients; their average PA concentration in the blood was 1.09. The difference between these means is not significant.

The erythrocyte sedimentation rate, determined on the same day as PA, was < 30 mm/hour in 30 patients; their average PA concentration in blood was 1.11 mg%. In 25 patients, the E.S.R. was ≥ 30 mm/hour, and the mean for blood PA concentration in these patients was 1.17 mg%.

The rheumatoid arthritis was estimated as belonging to *Stages I—II* in 29 cases; their average PA concentration in blood was 1.24 ± 0.063 mg%. In 26 cases the disease was classified as *Stages III—IV*, and the mean for blood PA concentration in these patients was 1.02 ± 0.064 mg%. The difference between these two means is probably significant ($T = 2.33$; $p < 0.05$).

At the time of the PA determinations, 19 patients were treated with sodium aurothiomalate; their average blood PA concentration

was 1.16 mg%. Sixteen patients were treated with chloroquine diphosphate alone and the mean for blood PA concentration in these patients was 1.17 mg%. Nine patients had received various kinds of treatment, and their average PA concentration in blood was 1.05 mg%. The differences between the means are not significant.

The average PA concentration in *synovial fluid* of 22 patients with rheumatoid arthritis was 1.52 ± 0.019 mg% (SD 0.088 and range 1.21—2.31). The difference between the average concentration of PA in blood and in synovial fluid is highly significant ($T = 7.25$; $p < 0.001$).

In 5 cases the synovial fluid PA was determined before and one after the intra-articular injection of 62.5 mg of hydrocortisone acetate. The results were as follows:

Subject	Synovial Fluid PA, mg%		Change
	Before Hydrocortisone	After Hydrocortisone	
S. A.	2.31	1.67	—0.64
K. K.	1.94	1.82	—0.12
E. J.	1.28	1.71	+0.43
H. H.	1.91	2.55	+0.64
A. R.	1.21	0.90	—0.31

The effect of intra-articularly injected hydrocortisone acetate on the synovial fluid PA concentration is, according to these results inconsistent.

DISCUSSION

As compared with the healthy individuals, the average concentration of PA in blood was increased in the patients with rheumatoid arthritis. Observations of similar (2, 10) and opposite (3) kinds have previously been made. In the last mentioned study, the PA determinations were performed on arterial blood and the group with joint symptoms consisted of 9 patients. The increase in blood PA in rheumatoid arthritis was of the same magnitude as in the patients with pneumonia, pleuritis, malignant tumours or thyrotoxicosis.

The duration of the rheumatoid history had no significant effect on the blood PA concentration. The urinary excretion of thiamine was observed to be slightly lower in the patients with rheumatoid arthritis and high erythrocyte sedimentation rate (6). The blood PA concentration was not influenced by the height of the E.S.R. The blood PA concentration is, according to our results, probably slightly lower in Stages III—IV than in Stages I—II. It has been previously observed that the extracellular fluid phase is greater in Stages III—IV than in Stages I—II (5). It is thus possible that the probably lowered values for blood PA in Stages III—IV are due to the greater dilution of PA in these, than in Stages I—II.

The patients treated with gold salts or chloroquine preparations did not differ as regards blood PA. It has been stated earlier that, during treatment with glucocorticoids, there may occur an accumulation of blood pyruvates (8, 7, 9). Our material did not include patients under glucocorticoid treatment.

The PA concentration in synovial fluid was significantly higher than the blood PA concentration. We have performed some PA analyses on the ascites fluid in peritoneal carcinosis and on the pleural fluid in patients with pleuritis. The PA concentration in these exudates was of the same magnitude as in the synovial fluid. It has been observed previously that intrathecally administered hydrocortisone causes a prompt decrease in the PA concentration in cerebrospinal fluid in patients with tuberculous or benign lymphocytic meningitis; in purulent meningitis, on the contrary, hydrocortisone has not the same constant effect (4). In our series of patients with rheumatoid arthritis, intra-articular hydrocortisone had no uniform effect on the synovial fluid PA concentration.

SUMMARY

1) The average blood pyruvic acid (PA) concentration in 20 healthy students was 0.83 ± 0.041 mg%. In 55 patients with definite rheumatoid arthritis the average was 1.15 ± 0.047 mg%. The difference between these means is significant ($p < 0.01$). The average blood PA in the pneumonia group was 1.22 mg%, in the pleuritis group 1.19 mg%, in the patients with malignant tumours 1.43 mg%, and in the thyrotoxicosis group 1.22 mg%.

2) The length of rheumatoid history or the height of the erythro-

cyte sedimentation rate had no significant effect on the blood PA concentration. In the patients belonging to Stages III—IV, the blood PA was probably lower ($p < 0.05$) than in Stages I—II.

3) The average PA concentration in synovial fluid in 22 patients with rheumatoid knee effusions was 1.52 ± 0.019 mg%. It is significantly higher than in blood ($p < 0.001$). The effect of intraarticularly administered hydrocortisone acetate on the synovial fluid PA concentration was not uniform.

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PYRUVICEMIA AFTER MYOCARDIAL INFARCTION

by

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It is known that serum glutamic pyruvic transaminase may increase after myocardial infarction (8, 5). In the present work we have endeavoured to follow the pyruvic acid concentration in blood after myocardial infarction.

MATERIAL AND METHODS

Our series consist of 19 patients (14 men, 5 women) treated in our Clinic for recent myocardial infarction.

The blood specimens for determination of pyruvic acid (PA) were taken at 8 a.m. after an overnight fast, and determinations immediately made. The method presented by Bonting (2) was employed, except that the volumes used were in millilitres, and the blood samples deproteinized by precipitation with trichloroacetic acid. With this method, the average blood PA concentration 0.83 ± 0.01 mg% (SD 0.184, range 0.53—1.29) was obtained in 20 healthy students.

RESULTS

The results are presented in Fig. 1. In 12 cases, increased PA values were distinctly seen in the first five days after infarction. The mean for the first PA value was 1.58%, for the second 1.21, and for the third 1.17 mg%.

¹ Aided by a grant from the Sigrid Jusélius Foundation.

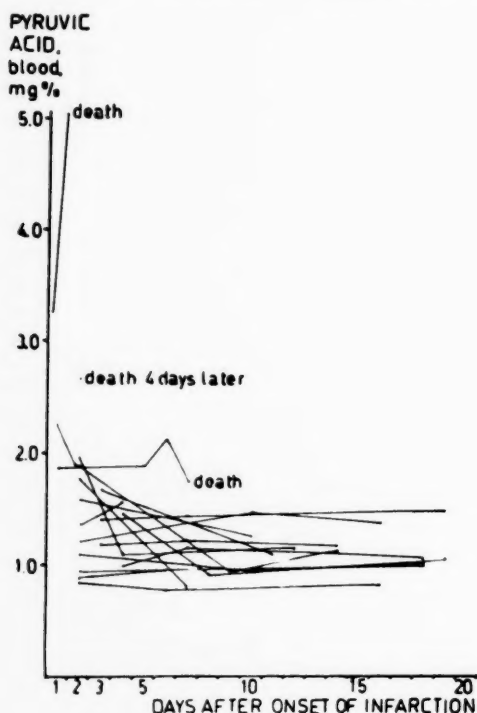


Fig. 1. — The pyruvic acid concentration in blood of 19 patients with myocardial infarction. The determinations of pyruvic acid were performed at various intervals after the onset of infarction.

In six cases the initial PA value in blood was over $1.80 \text{ mg}\%$. Three of these six patients died in the first week in hospital.

DISCUSSION

We have not succeeded in finding reports of a similar kind in the literature. Markees (7) enumerates the following diseases or conditions in which increased values for blood PA have been observed: acidosis, renal, hepatic and Basedowian coma, severe acute hepatitis, X-ray treatment, surgical operation and anesthesia, cardiac failure, congenital heart disease, anoxia, fever, infection, toxicosis of infancy, pregnancy, intoxications from sleeping medicines, dyspepsia of infancy, acetonemic vomitings, insulin shock, some endocrinic disturbances, and disseminated sclerosis. On the basis of our material, it has not been possible to

establish the primary cause of the increased PA values in blood during the first five days after the onset of myocardial infarction. Shock, anoxia, physical and emotional stress, tachycardia, fever and cardiac failure are conditions very often associated with increased PA in the blood (1, 3, 4, 6). All these factors play a special role also in the clinical picture of myocardial infarction, especially during the first days after the onset.

Determinations of the PA in blood have, to judge from our results, a limited diagnostic significance in patients with myocardial infarction. The very high values may, perhaps, have some prognostic significance.

SUMMARY

The pyruvic acid (PA) concentration in blood has been studied after the onset of myocardial infarction in 19 patients.

PA values over 1.30 mg% were seen in 12 patients during the first five days after the appearance of the infarction. The average PA concentration, determined for the first time after infarction (during the first 1—4 days), was 1.58 mg%; the mean for the second determinations was 1.21 mg% and for the third 1.17 mg%. Three of the six patients with an initial PA value over 1.80 mg% died in the first week in hospital.

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PYRUVIC ACID

ITS PRESENCE IN THE BLOOD, URINE AND GASTRIC JUICE OF
PATIENTS WITH PEPTIC ULCER AND ACHLORHYDRIA, AND IN PARTIALLY
GASTRECTOMIZED PATIENTS AND CARRIERS OF THE FISH TAPEWORM

by

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It has been previously reported from our Clinic that patients with achlorhydria, and partially gastrectomized patients, such with peptic ulcer, and carriers of fish tapeworm (*Diphyllobothrium latum*) may have disturbances in the urinary excretion of some vitamins belonging to the vitamin B group. The urinary excretion, especially of thiamine, was reduced in the patients with achlorhydria, in the partially gastrectomized patients and in the anemic carriers of tapeworm (2, 8).

In the present work we report our results concerning determinations of pyruvic acid in blood, urine and gastric juice in patients with achlorhydria, in partially gastrectomized patients, in peptic ulcer, and in carriers of fish tapeworm.

MATERIAL AND METHODS

The groups of patients subjected to our study are as follows:
Achlorhydria Group. — The pyruvic acid (PA) concentration in blood was studied in 10 patients, and the urinary excretion of PA was determined in 36 cases. In each case the 24-hour urine was

¹ Aided by a grant from the Sigrid Jusélius Foundation.

collected during three successive days and subjected to analysis of PA. Each individual value for urinary PA excretion in 24 hours is thus the mean of these three 24-hour urine samples. In 3 cases the PA was analysed in achlorhydric gastric juice. Achlorhydria was confirmed by twice performed histalog-tests.

Partially Gastrectomized Patients. — All were resected according to the method called Billroth II, and the indication for surgical operation was always peptic ulcer. The determinations of blood PA were performed in 18 patients; six of these had no acid secretion in the histalog-test, in 12 the gastric juice contained free hydrochloric acid (the position of a stomach tube was controlled by fluoroscopy). Urinary excretion of PA was determined in 40 patients; 20 of these had no acid secretion in the histalog-test and the remaining 20 were able to secrete free hydrochloric acid after the histalog provocation. The values for the urine PA are the means of the determinations performed on three successive 24-hour urine specimens. Gastric juices of partially gastrectomized patients were subjected to analysis of PA in nine instances.

Three partially gastrectomized patients underwent a three-week treatment with oral neomycine sulphate (1.65 g daily) and determinations of blood and urine PA were performed once a week during the period of treatment.

Peptic Ulcer Group. — Only the urinary excretion of PA was studied in this group. Collection of urine and calculations were done as above. The group includes 19 patients. All had acid gastric secretion.

Carriers of Fish Tapeworm. — Analysis of the blood PA content was performed in only six cases, and the urinary excretion of PA was followed in 25 patients; 5 of these carriers had fish tapeworm anemia. The gastric secretion was acid in 20 patients, and in 5, no acid secretion was obtained in the examination.

Controls. — Twenty healthy students were considered suitable for control of the blood PA determinations. Their gastric secretion status was not examined. Sixteen patients, hospitalized for coronary diseases, form a control group for the urine PA determinations. All of these had acid gastric secretion. In 7 cases, «normal» acid gastric juice obtained from patients without radiological or clinical gastric abnormalities or symptoms was subjected to analysis of PA.

The patients in all the above mentioned groups were hospitalized,

except the students. Specimens for blood or gastric juice determinations were taken in the morning. During the day, the patients were not completely bedridden.

Determination of PA was performed according to the method presented by Bonting (1) with the exception that the volumes used for determination were in millilitres, and deproteinization of the samples was performed by precipitation with trichloroacetic acid. The 24-hour urine samples were preserved before determination by adding 10 ml of conc. hydrochloric acid. To 2.0 ml of urine was added 150 mg of Lloyd's reagent, and thereafter the procedure was the same as for determinations in blood.

RESULTS

TABLE 1

RESULTS OF DETERMINATIONS OF PYRUVIC ACID IN BLOOD AND URINE OF PATIENTS WITH PEPTIC ULCER, IN ACHLORHYDRIA, IN PARTIALLY GASTRECTOMIZED PATIENTS, AND IN CARRIERS OF FISH TAPEWORM

Group	No. of Cases	Mean	Range	Standard Deviation	Standard Error of the Mean
<i>Blood, mg %:</i>					
Healthy students	20	0.83	0.53—1.29	0.18	0.04
Achlorhydria	10	1.09	0.71—1.75	0.29	0.09
Partially gastrectomized patients: acid stomach	12	1.11	0.72—1.46	0.21	0.06
achlorhydric stomach	6	1.26	0.86—1.65	0.29	0.12
total	18	1.16	0.72—1.65	0.25	0.06
Carriers of fish tapeworm	6	1.02	0.79—1.32	0.21	0.08
<i>Urine, mg/24 hrs:</i>					
Controls with acid stomach	16	17.1	6.7—48.0	10.5	2.6
Peptic ulcer	19	10.2	2.9—22.1	4.8	1.1
Achlorhydria	36	12.9	4.1—23.8	5.7	0.9
Partially gastrectomized patients: acid stomach	20	16.2	6.1—33.1	4.9	1.1
achlorhydric stomach	20	13.8	3.6—39.4	9.0	2.0
total	40	15.0	3.6—39.4	7.8	1.2
Carriers of fish tapeworm:					
anemic	5	9.8	3.7—13.9	3.9	1.8
non-anemic	20	12.5	5.2—18.1	3.3	0.7
total	25	12.0	3.7—18.1	3.6	0.7

The results of blood and urine PA determinations are presented in Table 1. It is seen that the blood PA concentration is significantly higher in patients with achlorhydria, in partially gastrectomized patients and in carriers of fish tapeworm, than in healthy students. The partially gastrectomized patients whose stomachs were capable of secreting free hydrochloric acid tend to have higher blood PA values than the partially gastrectomized patients without acid secretion in the stomach. The difference is not, however, statistically significant.

Regarding the urinary excretion of PA, presented in Table 1, it is seen that the patients with peptic ulcer and the anemic carriers of fish tapeworm have a significantly lower 24-hour output of PA than the controls. All other differences between the means given in the table are statistically insignificant.

The average concentration of PA in gastric juice was 0.83 mg% (range 0.14—1.68) in the «normal» cases, in achlorhydria 0.97 mg% (only 3 determinations — 0.47 mg%, 1.00 mg%, 1.46 mg%), and in the partially gastrectomized patients 0.96 mg% (range 0.62—1.45). The differences between the means are not significant.

The results of the blood and urine PA determinations during oral neomycine sulphate treatment in three partially gastrectomized patients were as follows:

Subject	PA	Before Neomycine	During Neomycine Treatment		
			1st Week	2nd Week	3rd Week
1.	Blood, mg % ..	1.44	0.94	0.93	1.05
	Urine, mg/24 hrs	7.7	4.2	5.6	5.4
2.	Blood	1.15	lacking	1.20	0.90
	Urine	17.6	*	17.4	16.2
3.	Blood	1.15	*	1.69	1.03
	Urine	13.4	*	10.2	12.1

The values obtained during the third week of neomycine treatment are in all three cases lower than before treatment. As to the urine values, the differences are, however, very small.

DISCUSSION

The results obtained with the method used for determinations in blood of healthy students agree well with those reported in the

literature (6, 4, 3). In their paper chromatographic analyses of PA, Kulonen *et al.* (5) obtained 16.9 mg for the average urinary excretion/24 hours. The corresponding mean for the control group in our material was 17.1 mg/24 hours. The primary cause of the increased concentration of PA in blood of patients with achlorhydria, in partially gastrectomized patients, and in carriers of fish tapeworm was not revealed in our material. It has been previously stated that the urinary excretion of thiamine is reduced in all these conditions (2, 7). It is known that deficiency of thiamine may cause an increase in blood PA (9). A contradictory finding is, however, the decreased urinary excretion of PA in peptic ulcer and in the anemic carriers of fish tapeworm. As mentioned earlier, in the patients with tapeworm anemia the urinary excretion of thiamine is reduced to one fourth of the normal (8); in peptic ulcer, on the other hand, the excretion of thiamine is normal or slightly decreased (7). It seems obvious that the urinary excretion of thiamine and PA are not quite closely associated. The correlation between blood and urine PA does not either seem to be good — as is seen from our results. It is interesting to observe also that the concentration of PA in the gastric juice was the same in a «normal» acid stomach, in a partially resected stomach, and in an achlorhydric stomach. This observation may arouse many theoretical problems which cannot be discussed on the basis of the present material.

SUMMARY

1) The average blood pyruvic acid (PA) concentration in 20 healthy students was 0.83 ± 0.041 mg%, in 10 patients with achlorhydria 1.09 ± 0.09 mg%, in 18 partially gastrectomized patients 1.16 ± 0.06 mg and in 6 carriers of fish tapeworm 1.02 ± 0.08 .

2) The average urinary excretion of PA (mg/24 hrs) was in 16 controls with acid gastric secretion 17.1 ± 2.6 mg, in 19 patients with peptic ulcer 10.2 ± 1.1 mg, in 36 achlorhydric patients 12.9 ± 0.9 mg, in 40 partially gastrectomized patients 15.0 ± 1.1 mg, in 5 patients with fish tapeworm anemia 9.8 ± 1.8 mg and in 20 non-anemic carriers of fish tapeworm 12.5 ± 0.7 mg.

3) The average content of PA in a «normal» acid stomach was

0.83 mg% (range 0.14—1.68), in achlorhydria 0.97 (range 0.47—1.46), and in a partially resected stomach 0.96 mg% (range 0.62—1.45).

4) During oral treatment with neomycine sulphate, slightly lower blood and urine values were obtained in the third week of treatment than before treatment.

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EFFECT OF DIETARY PROTEIN, METHIONINE AND CHOLINE ON ATHEROSCLEROSIS AND SERUM AND LIVER LIPIDS IN CHOLESTEROL-FED CHICKENS

by

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It was demonstrated in a previous study (10) that very low levels of protein in the diet enhance the hypercholesterolemia and atherosclerosis induced by cholesterol feeding in chickens. The present experiment was designed to study the effects of a diet less deficient in protein and of its supplementation with methionine and choline.

EXPERIMENTAL

One hundred and fifty 1 day old cockerels were randomly divided into six groups, 25 in each. All were given the same commercial chicken food without any supplements up to the age of 5 weeks. At this time the experimental diets were started. Group I continued with the «normal» diet, which contained 21 per cent of digestible protein, 5 per cent of fat, 46 per cent of carbohydrates (by weight) and sufficient amount of minerals and vitamins. Group II received a mixture of the above basal diet, potato starch and corn meal in a proportion to contain only 9 per cent of protein and correspondingly more carbohydrate. All the following groups were fed with this low protein ration supplemented by ingredients as follows: Group III, cholesterol 1.5 weight per cent mixed with the food as crystalline powder; group IV, cholesterol 1.5 per cent and methionine 1 per cent; group V, cholesterol 1.5 per cent and

choline chloride 1 per cent; group VI cholesterol 1.5 per cent, methionine 1 per cent and choline 1.5 per cent. All groups received food and water ad libitum. A new batch of food was prepared each week and, thus, the feed intake was easily controlled. This was, on an average, greatest with the normal diet (about 100 gm per chick per day), slightly smaller in the low protein groups and about one fourth smaller in the groups receiving choline supplement. The gain in weight paralleled the feed intake the average weight at the end of the experiment being 1720 gm in group I and 1460 gm in group V. All the birds surviving the experimental period were in vigorous health.

After a period of 12 to 13 weeks on experimental diets blood was drawn from the vein under wing and the birds sacrificed by decapitation. A sample of liver was removed for histological examination and another one for lipid analysis. The whole aorta, its main branches and the heart were removed *en bloc*, a careful evaluation of macroscopic atherosclerosis was carried out and specimens at three standard levels of the aorta were taken for histological inspection. The details of the method used for grading the lesions have been recently described (Nikkilä and Ollila 1959). This publication also gives the methods used in the chemical analyses.

RESULTS

A considerable number of birds died during the pre-experimental period and a few additional ones during the experiment leaving an unequal number of animals in each group (Table 1).

The results indicate that serum cholesterol level was not influenced by reduction of dietary protein in the absence of chole-

TABLE 1

SERUM CHOLESTEROL LEVEL AND THE INCIDENCE OF ATHEROMATOSIS IN THORACIC AORTA IN COCKERELS RECEIVING DIETS GIVEN IN FIGURES 1 AND 4

	I	II	III	IV	V	VI
Number	19	18	16	22	12	18
Serum cholesterol	100.9	112.6	377.9	198.2	536.7	225.6
(mean \pm s.d.)	± 34.1	± 24.2	± 221.0	± 91.8	± 366.1	± 99.5
Atheromatosis of thoracic aorta (per cent incidence)	11	37	87	60	83	67

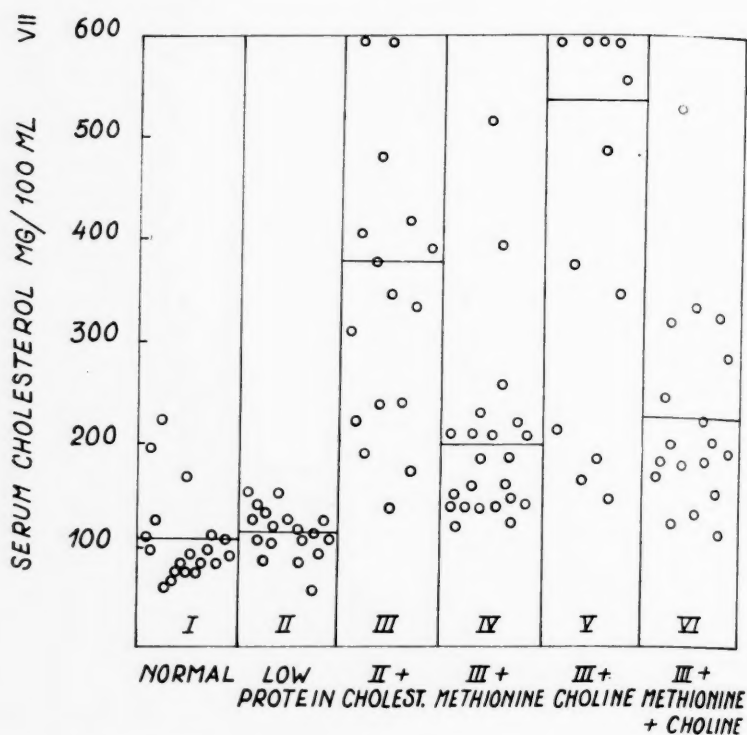


Fig. 1. — Serum cholesterol level in cockerels after 12 weeks on diets indicated.

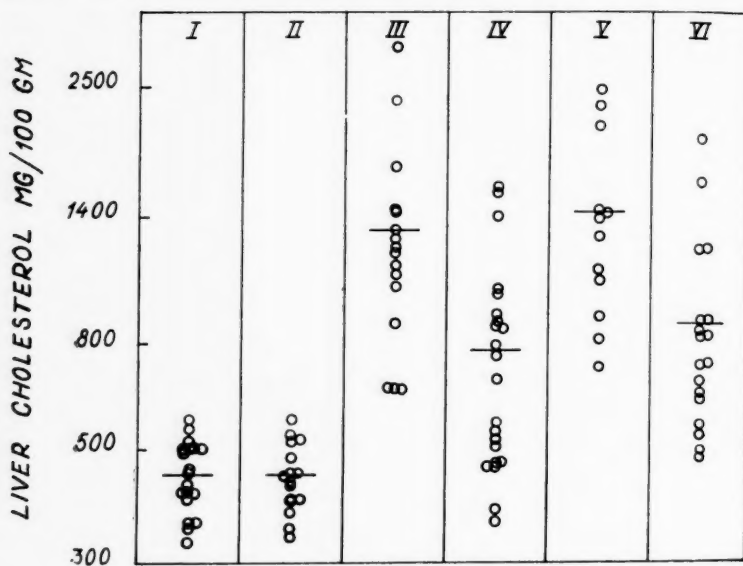


Fig. 2. — Liver cholesterol content (expressed per wet weight). Grouping as in figure 1.

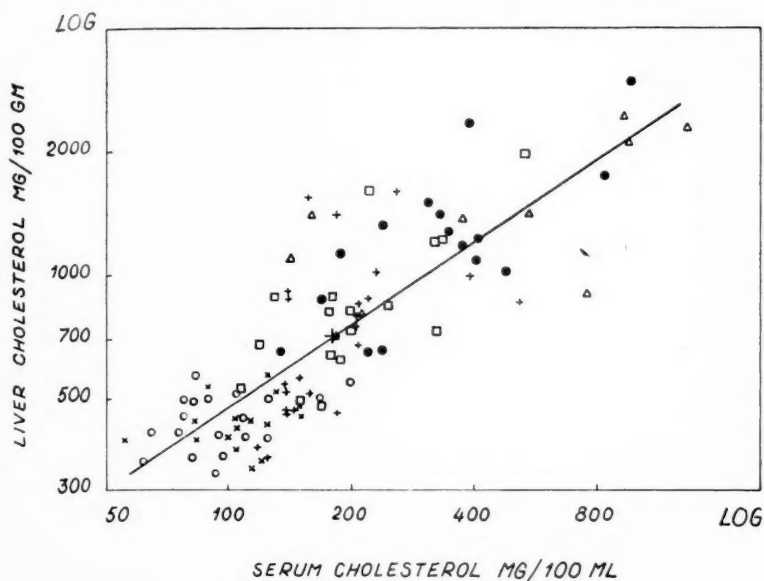


Fig. 3. — Correlation of serum and liver cholesterol. \circ = group I, \times = group II, \bullet = group III, $+$ = group IV, \triangle = group V, \square = group VI.

terol supplementation. On incorporation of cholesterol to the low protein diet the serum cholesterol content rose to about four-fold value. This increase was prevented by administration of methionine whereas choline was without effect (Fig. 1). The cholesterol level in the methionine-supplemented group was about equal to that in chickens fed cholesterol-supplemented normal diet (group 2 in previous study).

Similar differences were found in the cholesterol content of liver (Fig. 2) and a significant positive correlation exists between serum and liver cholesterol levels in the whole material ($r = +0.83$, $p < .001$) (Fig. 3).

The aortic atherogenesis paralleled the serum cholesterol only roughly (Table 1, Fig. 4) and the differences between the various treatment groups were not as distinct as in the cholesterol. The incidence and severity of lesions in abdominal aorta were slightly greater with low protein diet than with the normal one but statistically the difference remains insignificant. Supplementation of the low protein + cholesterol diet with methionine caused some decrease in the mean grade of atheromatosis of abdominal aorta.

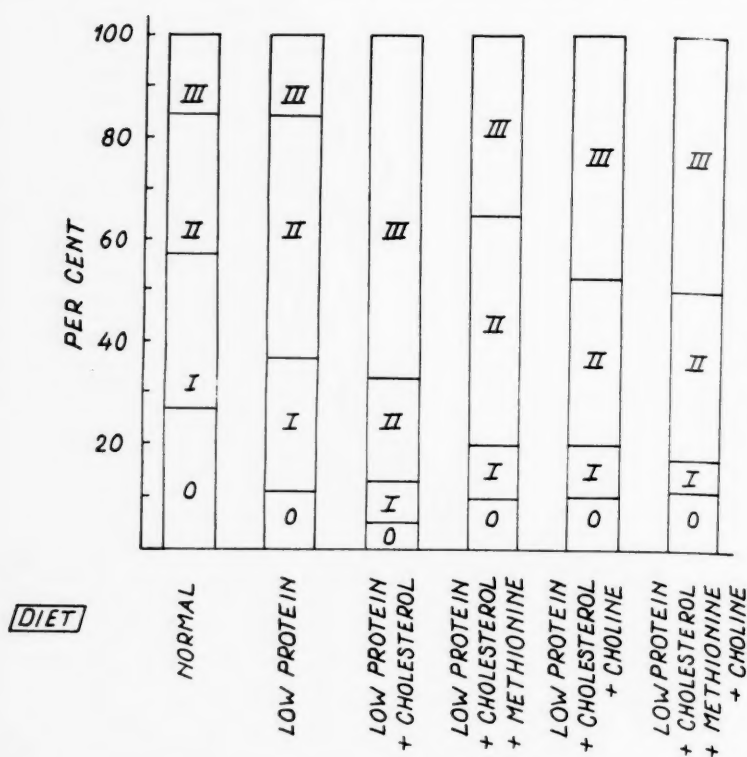


Fig. 4. — Grade of atheromatosis of abdominal aorta in different treatment groups. Grade II includes the grades 2 and 3 and grade III the grades 4 and 5 of our previous classification (Nikkilä and Ollila 1959).

The rather small differences in the involvement of abdominal aorta are partly explained by the exceptionally high incidence of spontaneous lesions in this series. In thoracic aorta the intergroup differences were more marked (Table 1). Only the incidence of macroscopic lesions was recorded here. Decrease of dietary protein per se caused a slight increase in atheromatosis of thoracic aorta and on adding cholesterol almost all birds became involved. A small decrease in incidence was again apparent in groups receiving the methionine supplement. In the whole series the correlation between serum cholesterol level and the grade of aortic atheromatosis was rather poor. It should be noted, however, that serum cholesterol determination was made only at the end of the experimental period.

DISCUSSION

The findings of the present experiment extend our previous observations (10) and demonstrate that the hypercholesteremia and cholesterol accumulation in the liver induced by cholesterol feeding in combination with low protein diet in cockerels is largely prevented by methionine administration. Simultaneously the aortic atherogenesis is retarded as compared to animals on cholesterol-low protein ration. These findings are in accord with the earlier results of Mann *et al.* (8) in monkeys and of Fillios and Mann (1) in rats and mice and they are further corroborated by the recent observations of Nishida *et al.* (12), Kokatnur *et al.* (5) and Stamler *et al.* (15, 16) in chicks.

Supplementation of low protein diet with choline revealed to be without effect on hypercholesteremia and atherosclerosis. This fact indicates that the methyl group is not involved in the action of methionine, a conclusion arrived at also by Mann *et al.* (8) and by Nishida *et al.* (12). The mechanism by which methionine counteracts the cholesterol accumulation remains to be clarified. In view of its parallel effects on liver and plasma cholesterol it seems plausible that a sulfur amino acid is essential for the biliary clearance of cholesterol converted to bile acids (10).

That differences in the incidence and severity of aortic atherosclerosis were not as marked as those in serum cholesterol level is probably due to the relatively long experimental period and due to the exceptionally high incidence of spontaneous lesions.

The main object in undertaking our previous and the present experiment was to elucidate the possible relationship of dietary proteins and amino acids to the development of atherosclerosis in man. Since that time a number of both animal and human investigations has been concerned with this problem but instead of having been clarified by these the question seems to have become only more confused by conflicting results. Some comments of these studies and of suggestions derived from them are presented below as an attempt to contribute receiving of a more clear picture of the matter.

Experiments carried out on dogs (7), monkeys (8), rats (1, 3) and chickens (2, 5, 6, 12, 15, 16) have uniformly shown that the hypercholesterolemia produced by fat and/or cholesterol supple-

mentation of diet is augmented by a simultaneous restriction of protein intake. In species susceptible to atherosclerosis this effect is reflected as increased atherogenesis as well, and both effects are counteracted by methionine administration whereas choline is ineffective. On the other hand, a diet abundant in protein may have similar effects as protein deficiency in cholesterol-fed rat (3) but not in chick (2, 12). The apparently opposite result of Olson *et al.* (13), viz., decrease of serum cholesterol and β -lipoprotein levels in rats on a soy protein diet deficient in methionine and choline, is probably explained by the fact that extra cholesterol or fat was not incorporated in the diet.

In man, evidence resting on well controlled dietary experiment is scanty. In subjects consuming a relatively low fat diet Keys and Anderson (4) could find no effect on serum cholesterol level of varying daily protein intake from 63 to 138 gm (at the expense of carbohydrate) and irrespective of whether cholesterol intake was low or high. In the experiment carried out by Olson *et al.* (14) total elimination of abimal protein from the diet of nine volunteers and its substitution with isocaloric amount of carbohydrate was followed by a decrease of serum cholesterol level averaging 44 mg per cent as compared to a relatively high protein intake. The daily fat consumption of these subjects was 80 gm (30 per cent of total calories) and the methionine contained in the low protein regimen apparently did not meet the minimal requirement. Supplementation of «normal» diet with methionine does not influence the serum lipid levels (Mann *et al.* 1953 b). Evidence provided by epidemiological data shows that the incidence of «arteriosclerotic heart disease» (certainly not an exact indicator of the incidence of coronary atherosclerosis) is to a certain extent correlated to the intake of animal protein but not to that of total protein (17, 18).

Discussion of the above results has, interesting enough, led to quite opposite suggestions on the role of dietary protein in human atherosclerosis. The significance of the results of Keys and Anderson (4) has been criticized both because of the high range of protein intake studied (14) and because of the relatively low fat intake of the subjects during the experiment (16). Both Nishida *et al.* (12) and Stamler *et al.* (16) have pointed to the possible significance in human atherosclerosis of the dietary fat to protein ratio and advanced the tentative hypothesis that among populations con-

suming high-calorie, high-fat diet the protein intake, though clearly sufficient *per se*, may be relatively inadequate. On the other hand, Olson *et al.* (14) hold the view, supported by their own experiments and by epidemiological data, that diets low in animal protein may have a favorable influence on human atherogenesis. While there is no direct evidence to disprove the former concept — the results of Keys and Anderson and of Olson *et al.* are not necessarily such — it has no convincing support either, not even in the own experiments of Nishida *et al.* and of Stamler *et al.* Thus, a closer examination of the data in both of these investigations reveals that in cockerels on high-fat high-cholesterol ration increase of the protein content of diet from the «normal» of about 20 per cent (by weight) to 30 or 35 per cent did not significantly reduce neither serum cholesterol level nor the severity of aortic or coronary atherosclerosis (the small differences found apparently do not reach the limit of statistical significance). Available human data do not strengthen the hypothesis either. Thus, the incidence of «arteriosclerotic heart disease» (according to vital statistics) in different countries does not correlate to the dietary protein/fat ratio better than to fat intake alone and no correlation at all is found between the former item and the dietary animal protein/total fat ratio. Because of the obvious unreliability of the present statistical data as indicators of coronary atherosclerosis this evidence is not necessarily very strong, however. On the other hand, the experimental conditions used by Olson *et al.*, *i.e.*, minimal protein intake combined to moderate fat consumption, hardly have a natural human parallel. To which extent and direction the serum cholesterol level is influenced by moderate variations in protein intake when fat provides some 30 to 40 per cent of total calories remains to be established by further dietary experiments in man.

SUMMARY

In continuation of an earlier investigation on the effect of decreased dietary protein on serum lipids and atherogenesis of cholesterol-fed cockerels the influence of supplementation of this diet with methionine and/or choline at 1 per cent level was studied. The diet was less deficient in protein (9 per cent) than in the previous study.

Methionine feeding almost completely abolished the potentiating effects of low protein intake on hypercholesterolemia, and liver cholesterol content and decreased the atherogenesis in cholesterol-fed cockerels whereas choline was ineffective in these respects. The significance of these and of previous experiments for human atherogenesis is discussed particularly from the point of view of the role of dietary protein in the development of the human disease.

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SERUM SEROMUCOID LEVEL IN EXPERIMENTAL ATHEROSCLEROSIS INDUCED BY CHOLESTEROL AND LOW PROTEIN DIET

by

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ESKO A. NIKKILÄ

(Received for publication January 9, 1960)

The role of connective tissue mucopolysaccharides in human atherogenesis was suggested several decades ago by the observations of Aschoff (3) and Hueck (10). According to Hueck the «mucoid degeneration» precedes all other changes. Schultz (18) demonstrated that the amount of metachromasia in and fatty degeneration of arterial wall are closely interrelated. More recent work has confirmed and extended these findings (9, 1, 17, 13, 23, 5). This knowledge has inspired studies on serum glycoproteins and seromucoid (mucoprotein) in human atherosclerosis. Elevated serum levels of both protein-bound hexose (24, 2, 6) and hexosamine (20) have been reported in this disease. According to Voigt & Schrader (24) α_2 -globulin-bound carbohydrate is increased in atherosclerosis more often than are *e.g.* serum cholesterol and lipoprotein levels. On the other hand, Pollak (16) was not able to demonstrate changes in the electrophoretic serum glycoprotein pattern in atherosclerosis. Seromucoid level has been found increased by DeFrancis *et al.* (6) and by Schwartz & Gilmore (20) whereas DiPaolo & Galletti (7) have reported, on an average, normal values. According to the studies of Schwartz & Casley-

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Smith (19) Australian aborigines living in primitive conditions had lower incidence of atherosclerosis and lower serum seromucoid levels than whites and urbanized aborigines. Also the serum haptoglobin concentration has been claimed to be increased in atherosclerosis (11).

Experimental atherosclerosis produced in animals by cholesterol feeding is also accompanied by changes in arterial connective tissue ground substance. This was suggested on the basis of rabbit experiments by one of us (14) and has since then been demonstrated both by histochemical methods (21) and by increased aortic uptake of radioactive sulfate in cholesterol-fed animals (4, 12). Schwartz *et al.* (21) found that hypercholesterolemia in rabbits was associated with elevated serum hexosamine levels.

On the basis of the above evidence plasma seromucoid has been studied in experimental atherosclerosis of cockerels.

MATERIAL AND METHODS

Cockerels used in this study represented a randomly selected sample of a larger series reported previously (15). The dietary treatment given to each of the six groups of birds is apparent from Table 1. For other details of the arrangement of the experiment reference is made to the above article.

The seromucoid was determined as protein by biuret reaction according to a modified Winzler method (22).

RESULTS

The results of seromucoid determinations as compared to mean cholesterol levels in different treatment groups are presented in Table 1. Low protein intake did not influence the seromucoid concentration whereas cholesterol feeding combined to this resulted in a significant increase of this serum component. This effect was inhibited by supplementation of the diet with both methionine and choline whereas neither of these compounds was effective alone. Thus, the alterations of seromucoid did not parallel those of serum cholesterol level though some degree of hypercholesterolemia seems to be a prerequisite for an increase of seromucoid fraction. The individual cholesterol and seromucoid levels did not

TABLE 1

SERUM SEROMUCOID AND CHOLESTEROL LEVELS IN COCKERELS AFTER 12 WEEKS ON DIETS INDICATED

Group	Diet	n	Seromucoid			Cholesterol Mean \pm s.e.
			Range	Mean \pm s.e.	p	
I	Control	13	80—214	161 \pm 8.6		92 \pm 5.4
II	Low protein	12	127—203	156 \pm 7.8	..	108 \pm 7.0
III	II + cholesterol	9	155—283	210 \pm 13.4	.01	467 \pm 90.0
IV	III + methionine	13	128—367	221 \pm 21.3	.02	176 \pm 12.1
V	III + choline	7	169—270	198 \pm 12.7	.05	568 \pm 161.7
VI	V + methionine	13	88—224	168 \pm 10.7	..	201 \pm 17.2

p = significance level of difference from control group.
s.e. = standard error of the mean.

show correlation with each other, nor was any strict correlation apparent between the grade of aortic atherogenesis and the seromucoid concentration.

DISCUSSION

The origin and metabolism of circulating seromucoid is too poorly understood to allow any conclusions to be drawn of its significance in atherogenesis. Thus, it is not known whether this acid carbohydrate-protein complex is released from tissue mucopolysaccharides and thus reflects the rate of depolymerization of the latter as suggested *e.g.* by Schwartz & Gilmore (20). More information on this equilibrium could possibly be provided by studies with S^{35} , which has been shown to be incorporated in serum α_1 -globulin as sulfate (8). The changes of arterial connective tissue (manifesting themselves *e.g.* as increased uptake of S^{35}) and of serum polysaccharide components found in experimental atherosclerosis are, directly or indirectly, secondary to the deranged lipid metabolism and suggest that this might apply also to the corresponding alterations found in the human disease. This simple fact seems to have been too little emphasized so far.

SUMMARY

Plasma seromucoid (mucoprotein) level was determined in 67 cockerels to study its possible correlation to atherosclerosis induced

by dietary means. Low protein cholesterol rich diet produced an increase of both serum cholesterol and seromucoid levels. The former effect was abolished by methionine administration whereas seromucoid was not affected. The seromucoid level was correlated neither with plasma cholesterol nor with the severity of aortic atheromatosis.

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COMPARISON BETWEEN THE EFFECTS OF PORK FAT
AND SAFFLOWER OIL ON SERUM LIPIDS AND ATHERO-
SCLEROSIS IN CHOLESTEROL-FED CHICKENS¹

by

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(Received for publication January 15, 1960)

The decreasing effect on serum cholesterol and phospholipid levels of substituting polyunsaturated fats in the diet for saturated ones is well recognized in man. Whether this is followed by a retardation of atherogenesis is not known for the present. Corresponding experiments in animals have given divergent results (1, 3, 5, 6, 7, 13, 14, 15, 16, 18, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30) as regards the relationship of dietary fat and serum cholesterol level. In almost all of these experimental studies coconut oil has been used as a representative of the group of saturated fats in spite of the fact that its fatty acid composition is far from characteristic to an average diet of «western» man. This oil contains up to 80 per cent of short-chain ($\leq C_{14}$) saturated fatty acids and is almost devoid of unsaturated ones whereas of the fatty acids contained, *e.g.*, in an average Finnish diet approximately only 10 per cent are of the former group but 40 per cent of the latter. This difference may be particularly significant as saturated fatty acids with short and intermediate chain length have been reported to have greater cholesterol-increasing activity than those with long chain (2), and, on the other hand, oleic acid (the main unsaturated fatty acid component of average human diet) is claimed to be

¹ Aided by a grant from Sigrid Jusélius Foundation, Helsinki, Finland.

indifferent in serum cholesterol regulation (12). These facts make the results of coconut oil experiments poorly applicable to human conditions.

The present study in cockerels was designed to compare the effects on serum lipids and atherosclerosis of diets containing (a) a fat resembling in composition the «average normal» dietary fat of the majority of «western» people and (b) a fat providing amply polyunsaturated fatty acids and little the saturated ones. Choice was made of pork fat and safflower oil as representatives of the above groups. The former contains (glycerol omitted) roughly 40 per cent of C_{16} — C_{18} saturated fatty acids, 50 per cent of monoenes (oleic) and 6 per cent of polyenes (linoleic) and is a relatively common food fat at least in Finland. Of the fatty acids of the safflower oil about 70 per cent are polyenes (linoleic) and only 10 per cent saturated ones.

EXPERIMENTAL

Ninety 1 day old chickens were randomly divided into three groups. All received the same commercial chicken food until 6 weeks of age. At this time the following experimental diets were started: group I stock diet supplemented with 1.5 per cent cholesterol; group II stock diet to which was added 10 per cent finely ground fresh pork fat plus 1.5 per cent cholesterol; group III stock diet supplemented with 10 per cent emulsified safflower oil plus 1.5 per cent cholesterol. The ingredients were completely mixed with the stock food in large blenders of a slaughterhouse refinery. Any selection of the individual food components was seemingly impossible. The stock diet contained 4 per cent crude fat, 18 per cent digestible protein and sufficient amounts of salts and vitamins. All birds received food and water ad libitum. No essential difference was discernible between the groups in the amount of food consumption.

Some chickens died during the pre-experimental period and some additional ones had to be excluded from the experiment after they turned out to be hens. This elimination leaved an unequal number of birds in each group (Table 1). The animals which completed the experimental period of 17 weeks were all in vigorous health.

The gain in weight was about equal in all groups the terminal average weight being as follows: Group I 1700 ± 350 g, Group II 1760 ± 280 g, and Group III 1760 ± 150 g.

After 5 and 11 weeks on experimental diets plasma cholesterol level was determined in 10 randomly selected cockerels of each group (the same animals being studied at each time). At the end of the experiment venous blood samples were drawn for lipid analysis (overnight fast) and the birds sacrificed by decapitation. In postmortem examination the presence and grade of atherosclerosis in aorta and its main branches were estimated according to the principles described earlier (19). Thyroid gland and liver were removed for histological evaluation. Plasma cholesterol and phospholipid were analyzed by the earlier established methods; neutral fat was determined as glycerol according to Van Handel & Zilver-smit (32).

RESULTS

The response of plasma cholesterol value to the dietary treatments given is illustrated in Fig. 1 and the average serum levels of the three lipids at the end of the experiment are included in Table 1. Throughout the experimental period mean plasma chole-

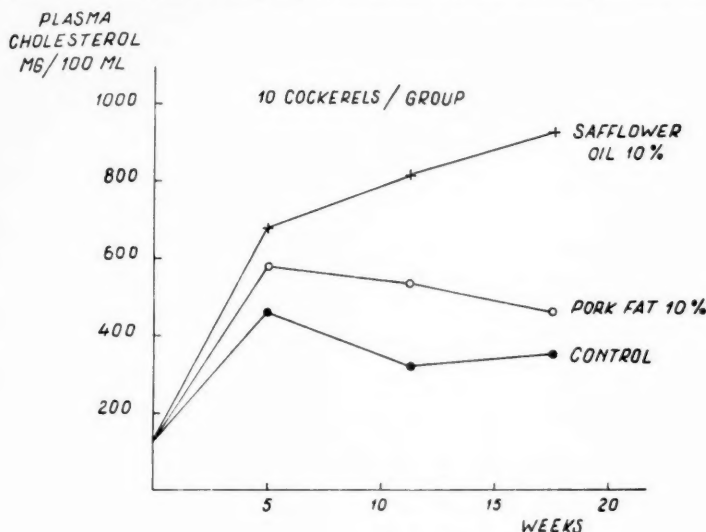


Fig. 1. — Mean plasma cholesterol in ten randomly selected cockerels receiving stock diet and the supplements indicated.

TABLE 1
AVERAGE PLASMA LIPID LEVELS AND STANDARD DEVIATIONS AFTER 17 WEEKS
ON EXPERIMENTAL DIETS

Dietary Supplement	n	Cholesterol mg/100 ml	P-lipid mg/100 ml	Neutral fat mg/100 ml
Cholesterol 1.5 % (C)	25	397 ± 132	237 ± 90	157 ± 62
C + pork fat 10 %	29	560 ± 279	274 ± 77	155 ± 44
C + safflower oil 10 %	19	884 ± 254	362 ± 67	188 ± 44

terol level was higher in fat-cholesterol fed birds than in those receiving cholesterol without added fat. The animals consuming safflower oil developed, in addition, consistently greater hypercholesteremia than those fed pork fat. Parallel but less marked changes were present in plasma phospholipid level (difference control-pork fat not significant) whereas the neutral fat content of fasting serum was unaffected by the fat feeding.

The incidence and severity of atheromatous involvement of the aorta roughly paralleled the serum cholesterol levels (Table 2).

TABLE 2
DISTRIBUTION OF BIRDS ACCORDING TO THE GRADE OF AORTIC ATHEROSCLEROSIS
AT THE END OF EXPERIMENT

	Abdominal aorta						Thoracic aorta		
	0	1	2	3	4	5	None	Slight	Marked
Cholesterol (C)	4	6	6	3	1	0	18	2	0
C + pork fat	3	3	11	6	4	0	19	7	1
C + safflower oil	1	2	4	4	5	1	3	4	10

The mean grade of atheromatosis of abdominal aorta (grading scale 0—5) was 1.5 in the control group, 2.2 in the group fed with pork fat and 2.8 in the safflower oil group. Comparison of the frequencies (pooled for grades 0—2 and 3—5) by chi-square test did not indicate any significant differences, however, between the three groups. In the thoracic portion of aorta the safflower oil fed birds showed significantly more atheromatosis than those receiving cholesterol alone or cholesterol-pork fat regimen. Though there was a trend to increased aortic atheromatosis with increasing hypercholesteremia the great variance of serum cholesterol level within each severity class of atherosclerosis made the regression of

atheromatosis on cholesterol to remain insignificant in all other groups except in the safflower oil one. In this group A (thor) + 1 = $4.55 \log C - 11.46$.

The histology of the liver was normal in group I, showed some fatty degeneration in one-fourth of the animals of group II (pork fat) and of parenchymal cells in a few of group III (oil). The average relative amounts of colloid and epithelium in thyroid glands were similar in all groups.

DISCUSSION

Whatever the results of the animal experiments are, they do not, of course, challenge the findings obtained in man. It would be erroneous, however, to deny all significance for human pathology of the information derived from experiments with animals. Much difficulties but lie in an attempt to realize which findings are pertinent to human disease and which are not. This is particularly true in experimental hypercholesteremia and atherosclerosis as strikingly demonstrated by the present experiment, which has given results seemingly contradictory to experience in man. An animal with diet-induced hypercholesteremia is well suited to search for factors causing uncoupling of the serum cholesterol-atherosclerosis relationship but is not an ideal tool in the study of cholesterol metabolism, because of the unphysiologically large cholesterol load.

The present experiment indicates that in chick a diet rich in polyunsaturated fatty acids and the accompanying high plasma and tissue concentration of these acids is ineffective against the arterial lipid deposition at any given plasma cholesterol level. This finding probably applies also to man. On the contrary, the augmenting effect on hypercholesteremia of oil feeding as compared to saturated fat is opposite to observations in man and also in some animal species (1, 3, 13, 14, 22, 25). The controversy is not easy to explain in spite of the fact that unsaturated fatty acids have been shown both to facilitate cholesterol absorption (31) and to stimulate its synthesis in the liver (3, 17). In any case rich dietary supply of polyunsaturated fatty acids does not prevent the development of hypercholesteremia when cholesterol intake is large.

The exact mechanism by which plasma cholesterol level decreases

on increased intake of polyunsaturated fatty acids in man is not known. Evidence has been presented for an increased fecal excretion of sterols (8, 17) and for a redistribution of cholesterol in the liver-plasma pool (3) but not for a decreased rate of synthesis. From the point of view of atherogenesis the redistribution thesis is important because exchange of cholesterol occurs not only between the plasma and liver compartments but also, though at a much slower rate, *e.g.*, between plasma and arterial wall.

Increased fecal output of sterols means a decreased absorption of cholesterol or its metabolic products including the dietary cholesterol. The generally held view that plasma cholesterol level is not influenced by dietary cholesterol within physiological range (9, 10) has not been supported by all recent experimental data (4).

SUMMARY

In cockerels fed cholesterol at 1.5 per cent level the diet was supplemented with pork fat or safflower oil (10 per cent) to study the effect of the dietary fat composition on serum lipids and atherogenesis. Both fats augmented hypercholesteremia and atherosclerosis as compared to cholesterol-fed controls without added dietary fat. The effect was greater with the safflower oil than with the pork fat. Thus, ingestion of a large amount of polyunsaturated fat does not inhibit atherogenesis induced by hypercholesteremia.

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EFFECTS OF LONG-TERM USE OF ANTIDIABETIC SULFONYLUREAS ON THYROID WEIGHT AND ARTERIOSCLEROSIS¹

by

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(Received for publication January 25, 1960)

The antidiabetic sulfonylurea compounds are known to have thiouracil-like antithyroid activity (2, 3, 6). This is too weak, however, to cause at the clinically effective dose level any signs of suppression in the conventional thyroid function tests even after long-continued use (4). However, in some patients a definite increase of the thyroidal radioiodine uptake occurs after withdrawal of the antidiabetic drug (4) and this suggests the possibility that these compounds might act as goitrogens particularly in areas with low dietary supply of iodine. To test this hypothesis the thyroid weights of sulfonylurea-treated diabetics were determined in an autopsy material and compared to thyroids of other diabetic cases. Because carbutamide has, in addition, been reported to increase serum cholesterol level in rats (1) and to enhance hypercholesteremia and atherosclerosis in cholesterol-fed cockerels (5) the degree of atherosclerosis (or combined athero-arteriosclerosis) was simultaneously recorded.

MATERIAL AND METHODS

The series includes all cases with diabetes mellitus autopsied at the Department of Pathology, University of Helsinki, during

¹ Aided by a grant from Sigrid Jusélius Foundation

the three-year period 1957—1959. No consideration was paid to the co-existent diseases or to the cause of death. The series numbering 72 cases was divided into three subgroups according to the manner of antidiabetic treatment they had received. To the *sulfonylurea* group (22 cases) were accepted all the patients, who had been treated with these drugs continuously for a minimum period of 6 months before their death. In the majority of cases the compound used was carbutamide and the average duration of treatment was about two years. The mean age of the members of this group was 73.2 years. The *insulin* group comprised 26 cases, which had regularly used insulin for at least 6 months. Their age averaged 55.6 years. The remaining 24 cases formed a somewhat heterogeneous «diet» group composed of patients, whose diabetes had been treated by dietary means only, who had received no treatment at all or who had used sulfonylureas or insulin for short periods. The mean age in this group was 71.4 years.

The groups did not significantly differ from each other in regard of sex distribution. The degree of control of the diabetes was difficult to assess because information of it was in general available only for a short period preceding the death.

The weight of the thyroid gland and the estimated degree of athero-arteriosclerosis in aorta and coronary and cerebral arteries were recorded from the autopsy protocols. Because of its retrospective nature the study has some inherent shortcomings. The thyroids had not been systematically weighed and the possibility exists that some selection had occurred the weighing being omitted more often in glands of obviously normal size than in clearly enlarged glands. The sulfonylurea and «diet» groups are apparently comparable in this respect, however. The sclerotic involvement of the arteries was graded from 0 to 3 according to the information given in the protocols. The degrees are rough estimates only, based on macroscopic evaluation. The grade 3 means several large calcified and often ulcerating plaques in the aorta and severe narrowing or complete occlusion in the cerebral and coronary arteries. Vessels with several atheromas but without occlusion were classified as grade 2 and those with only occasional shallow patches were regarded as grade 1 lesions. Records to allow classification according to this system were available in all cases

RESULTS AND DISCUSSION

The mean thyroid weight and the average grades of sclerosis of aorta and of coronary and cerebral arteries are given in Table 1. The size of the thyroid gland was not significantly different in the three treatment groups. The sclerosis was most advanced in the sulfonylurea group in all three arterial systems examined and least severe in the insulin group even after exclusion of the younger subjects. When tested by chi-square all intergroup differences remained insignificant, however.

On inspecting the collected data the impression was obtained that sclerosis was more advanced in cases with small thyroid glands than in those with goiters. For this reason a comparison was made on the grade of sclerosis in cases with the thyroid gland weighing less than 30 g and in those with thyroid weight exceeding 50 g without regard of the treatment group (Table 2). Testing

TABLE 1
MEAN WEIGHT OF THYROID GLAND (\pm S.D.) AND AVERAGE ESTIMATED GRADE OF AORTIC, CORONARY AND CEREBRAL ATHERO-ARTERIOSCLEROSIS IN DIABETICS.

(n refers only to the thyroid weight)

Treatment	n	Thyroid	Sclerosis, Graded 0-3		
			Aorta	Coron.	Cerebral
Diet	21	49.6 \pm 42.5	2.0	2.0	1.8
Sulfonylurea.....	16	52.3 \pm 43.0	2.5	2.5	2.1
Insulin	12	45.5 \pm 21.7	1.5	1.7	1.3
			1.9*	1.8*	2.0*

* Subjects of the insulin group over 55 years of age.

TABLE 2
ESTIMATED AORTIC, CORONARY AND CEREBRAL ARTERIOSCLEROSIS IN DIABETIC CASES WITH LARGE AND SMALL THYROID GLANDS

	n	Grade of Arteriosclerosis				
		0	1	2	3	Mean
<i>Thyroid weight > 50 g</i>	15					
Aorta.....		3	2	4	6	1.9
Coronary arteries		3	3	2	7	1.9
Cerebral arteries		3	2	5	5	1.8
<i>Thyroid weight < 30 g</i>	15					
Aorta.....		0	1	3	11	2.7
Coronary arteries		0	0	4	11	2.7
Cerebral arteries		0	4	3	8	2.3

of the differences by chi-square method (pooled grades 0—1 and 2—3) showed that subjects with small thyroids had significantly more coronary sclerosis than those with goiter ($P < 0.01$) whereas the differences in aorta and cerebral arteries could not be statistically verified. This result was not due to different age distribution.

Thus, the present study could not provide evidence for a goitrogenic action of sulfonylureas (mainly carbutamide) when used even for long periods at effective antidiabetic dose level. The result is not unequivocal, however, because it is based on a survey of routine autopsy records and the histological examination of thyroids has not been systematically performed.

On drawing any conclusions or making generalizations from the observed difference in coronary sclerosis between subjects with large and small thyroid glands one should realize that the series comprised only diabetics with a high age and, accordingly, with a high overall incidence of advanced arteriosclerosis.

SUMMARY

A survey of autopsy records of 72 diabetic patients was performed to determine the possible effect of long-term sulfonylurea treatment on the thyroid gland and the grade of aortic coronary and cerebral athero-arteriosclerosis. No essential difference was revealed in the weight of thyroid gland between diabetics who had been treated with sulfonylureas, insulin or with diet only. The sclerosis was most advanced in the sulfonylurea group but the difference from the others was not significant. Cases, pooled from all three treatment groups, with enlarged thyroids (over 50 g) showed significantly less coronary sclerosis than those with small thyroids (below 30 g).

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DIURETICS AND QUANTITATIVE HISTOENZYMOLOGY OF RAT KIDNEY

by

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The mechanism and locus of action of diuretics is only partially known. There is, however, ample evidence that diuretics interfere with certain enzyme systems controlling the tubular transport of urinary constituents. Best known is the inhibitory effect of acetazolamide on carbonic anhydrase, which regulates the renal reabsorption of bicarbonate and the elimination of anions bound with hydrogen, potassium and ammonium (7). These ion-exchange mechanisms contribute at least 10 per cent to the catalyzed reabsorption of sodium (1). The exact tubular level of carbonic anhydrase inhibition by acetazolamide is unknown, and it cannot be determined by the unspecific histochemical methods for carbonic anhydrase hitherto proposed (17).

About 90 per cent of the renal conservation of sodium is accomplished by the reabsorption of sodium with fixed anion against a concentration gradient. This active process requires energy, which is derived principally from the oxidative catabolism through the cycle of Krebs. On a weight basis, the consumption of oxygen and the content of the intermediary products of Krebs cycle is higher in kidney than in any other organ (5). The enzymes of this cycle seem to be the logical targets of diuretics, which produce diuresis by promoting the excretion of sodium. Mercurials and chlorothiazides are the most effective saluretics. Their effects are additive, and, therefore, it is suggested that mercurials and

chlorothiazides probably interfere with different enzyme reactions supplying energy to a single reabsorptive mechanism (20). The enzyme targets of chlorothiazides, other than carbonic anhydrase, are unknown. Mercury, on the other hand, is known to block sulfhydryl groups, which are required for the operation of several enzymes. The inhibition of succinic dehydrogenase in rat kidney by the administration of mercurial diuretics was first demonstrated manometrically by Handley and Lavik (8), and histochemically by Mustakallio and Telkkä (14). The common site of mercurial action of six diuretics and sublimate was found to be the straight terminal portion of the proximal convoluted tubule. The different organic carriers of mercury modified somewhat the involvement of other tubular segments presumably by influencing the excretion pathway of the diuretics (15). At corresponding sites, a histochemically demonstrable reduction in sulfhydryl groups was found (16). In contrast, acetazolamide and the chlorothiazides did not cause a histochemically demonstrable inhibition of succinic dehydrogenase and sulfhydryl groups (16).

The recent development of histochemical methods for pyridine-nucleotide coenzyme linked dehydrogenases (10) made the present extension in the study of diuretic action feasible. The histochemical results reported in this paper are correlated with quantitative enzyme data from four zones of rat kidney. Since the quantitative zonal histoenzymology of rat kidney has been hitherto relatively little explored (11), phosphatases and several enzymes lacking conventional histochemical staining methods have been included in this investigation.

MATERIAL AND METHODS

30 male albino rats of the Wistar strain weighing 200 to 270 g were used in the experiments. Water and food was provided *ad libitum*. The mercurial diuretics meralluride (Mercardac, Parke & Davis), mercaptomerin (Thiomerin, Wyeth), Esidron (Ciba), and mercuriophylline (Novurit, Medica) were administered subcutaneously in doses corresponding 10, 20 and 30 mg of mercury per kilogram of body weight. The subcutaneous dose of acetazolamide (Diamox inject., Lederle) was 100 mg per kg of body weight, and the same dose of dihydrochlorothiazide (Esidrex, Ciba) was administered by intubation. The diuretics were given at 23 o'clock. After 9 hours the rats were killed by decapitation together with pair fed littermates serving as controls. A piece from the immediately removed kidney was sectioned

simultaneously with a corresponding control specimen with a cryostat-microtome at 5 microns. Both sections were picked up on a warm (room temperature) coverslip and flooded immediately with the incubation mixture in a thermostat at 38°C. Nitro-Blue Tetrazolium was employed as the electron acceptor for the pyridinenucleotide coenzyme linked dehydrogenases, which were demonstrated according to the methods of Hess *et al.* (10). The histochemical method for succinic dehydrogenase (18) was modified by osmotic protection with 7.5 per cent polyvinylpyrrolidone Adenosine triphosphatase¹ was demonstrated after Padykula and Herman (19).

The contralateral kidney was dissected with scalpels into four zones on an inverted ice-dish. Zone I (inner medulla) consists of the single white papilla containing thin limbs of Henle's loops, collecting ducts, capillaries and fusiform interstitial cells. Zone II (outer medulla) which is pink in the fresh kidney, contains thin limbs, thick ascending limbs of Henle's loops, collecting tubules, and bundles of capillaries. Zone III (inner cortex) is made up principally of terminal portions of proximal convoluted tubules with well-delineated brush borders. Ascending thick limbs and collecting tubules traverse this zone individually. Zone IV (outer cortex) contains the glomeruli and the convolutions of proximal and distal convoluted tubules. The high cells of proximal convolutions bulge into the lumen in contrast to the relatively low cells of distal convoluted tubules, which have an intimate relationship to macula densa. Zones III and IV interdigitate in a saw-tooth fashion, which could not be adequately followed when the rapid dissection technique was employed. Fig. 6 A illustrates best the clearcut zonal pattern of rat kidney.

For the chemical enzyme assays each kidney zone was weighed immediately after dissection and homogenized in a cooled Waring blender with distilled water (1 ml for 10 mg of tissue). The homogenates were kept in ice and the enzyme determinations were carried out within the next few hours.

The assay mixtures of the DPN- and TPN-linked reactions are shown in Table 1. The assays were carried out in duplicate, each sample was allowed to stand for 10 minutes at room temperature before the substrate addition in order to minimize side reactions. Readings were taken at 30 second intervals at 340 m μ in a Beckman B spectrophotometer against a blank, where all reagents, except the substrate, were added.

The assay mixtures for the phosphatase determinations are presented in Table 2. The incubation was carried out for 30 minutes at two different

¹ The following abbreviations are used. DPN = diphosphopyridine nucleotide, TPN = triphosphopyridine nucleotide, G-6-PD = glucose-6-phosphate dehydrogenase, ICD = isocitric dehydrogenase, LDH = lactic dehydrogenase, MDH = malic dehydrogenase, α -GPDH = α -glycerophosphate dehydrogenase, β -HBDH = β -hydroxybutyrate dehydrogenase, SDH = succinic dehydrogenase, GLUT DH = glutamic dehydrogenase, P-ase = glycerophosphatase, PP-ase = pyrophosphatase, ATP-ase = adenosine triphosphatase, β -GLUC = β -glucuronidase, ALD = diphosphofructaldolase, ISOM = phosphohexose isomerase, GOT = glutamic oxalacetic transaminase.

TABLE 1

REACTION MIXTURES OF THE DPN- AND TPN-DEPENDENT ENZYME ASSAYS.
THE FINAL VOLUME OF EACH SAMPLE WAS 3 ML

Enzyme	Ml of Homogenate	Buffer	Cysteine	Activator	Coenzyme	Substrate
G-6-PD	0.5	tris, pH 7.5 0.03 M	0.005 M	MgCl ₂ 0.007 M	TPNH 4×10^{-5} TPN 1×10^{-4} M	glucose-6-phosphate 0.007 M
ICD	0.1	"	"	MnCl ₂ 0.0014 M	"	isocitrate 0.001 M
GR	0.1	"	"		TPNH 10^{-4} M TPN 2×10^{-5} M	glutathione, oxidized 10^{-4}
LDH	0.1	"	"		DPNH 2.5×10^{-4} M	pyruvate 0.003 M
MDH	0.05	"	"		"	oxaloacetate 0.001 M
GOT	0.05	phosphate pH 7.4, 0.08 M		malic DH	"	aspartate 0.035 M
ALD	0.3	collidine pH 7.4, 0.04 M		glycerophosphate dehydrogenase-triose-phosphate isomerase	"	alpha keto-glutarate 0.007 M fructose-1,6-diphosphate 0.0013 M

The reagents were commercial products from C. F. Boehringer & Sohne, Mannheim, Germany, from AG. Fluka, Basle, Switzerland, from California Foundation for Biochemical Research, Los Angeles, and from Sigma Chemical, St. Louis, USA.

TABLE 2

REACTION MIXTURES OF THE PHOSPHATASE ASSAYS IN A FINAL VOLUME OF
0.5 ML

Enzyme	Homogenate ml	Buffer	Activator	Substrate
P-ase	0.1	acetate pH 5.6, 0.04 M glycylglycine pH 9.4, 0.04 M	MgCl ₂ , 0.001 M	sodium glycerophosphate, 0.02 M
PP-ase	0.025	acetate pH 5.6, 0.04 M glycylglycine pH 9.0, 0.04 M	MgCl ₂ 0.0002 M	sodium pyrophosphate 0.0004 M
ATP-ase	0.1	acetate pH 5.6, 0.04 M glycylglycine pH 9.0, 0.04 M	MgCl ₂ 0.01 M	adenosine triphosphate, sodium salt, 0.003 M

pH values, corresponding to the activity maxima. In the blank samples incubation was omitted. Inorganic phosphorus was determined by the Fiske-Subbarow procedure.

The phosphohexose isomerase activity was determined according to Bodansky (2) from 0.05 ml of the homogenate.

In the β -glucuronidase assay phenolphthalein monoglucuronide was employed as substrate and the amount of phenolphthalein liberated by 0.1 ml of the homogenate during 30 minutes' incubation at pH 7.5 and 37°C, was determined colorimetrically at 550 m μ .

The tyrosine content of the homogenates, as determined by a modified method of Lowry *et al.* (12), was taken as a measure of soluble protein and used as reference basis for the enzyme activities.

RESULTS

Fig. 1 A to 7 A depict the distribution of the TPN- and DPN-linked dehydrogenases in rat kidney. Since the limited space does not permit a detailed description, the reader is referred to the investigation of Hess *et al.* (10). Our observations concur with their results in other respects except in the distribution of β -HBDH activity. They described Grade 4 and 2 β -HBDH activity for the terminal portion of proximal tubule and for the ascending thick limb of Henle, respectively, whereas in our preparations the corresponding activities were about *vice versa*. When substrates were omitted from the incubation mixtures, there was no blank staining for G-6-PD and for β -HBDH and a slight staining for the other dehydrogenases corresponding in distribution to the respective diaphorases. Fig. 8 A illustrates the distribution of alkaline ATP-ase. For details the reader is referred to the paper of Padykula and Herman (19).

Fig. 1 B to 8 B depict the histochemically demonstrable effect of mercurial diuretics on the corresponding enzymes. The sensitivity of the enzymes for mercurial inhibition decreased in the following order: SDH, β -HBDH, ATP-ase in the mitochondria and cell membranes, ICD, G-6-PD, α -GPDH, GLUT DH, MDH, and LDH. Irrespective of the enzyme or mercurial studied, the main site of enzyme inhibition was the straight terminal portion of proximal convoluted tubule. The extent of involvement of the proximal convoluted tubule seems to characterize each of the mercurial diuretics studied. For details the reader is referred to the investigation of Mustakallio and Telkkä (15). In the osmotically protected

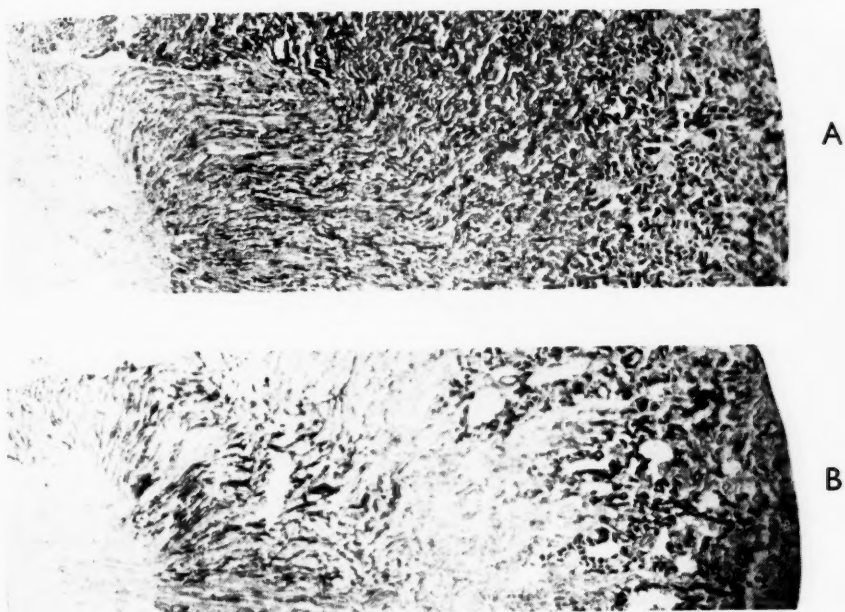


Fig. 1. — Histochemistry of G-6-PD
A Control (incubation 90 min)
B After meralluride, 20 mg/kg.

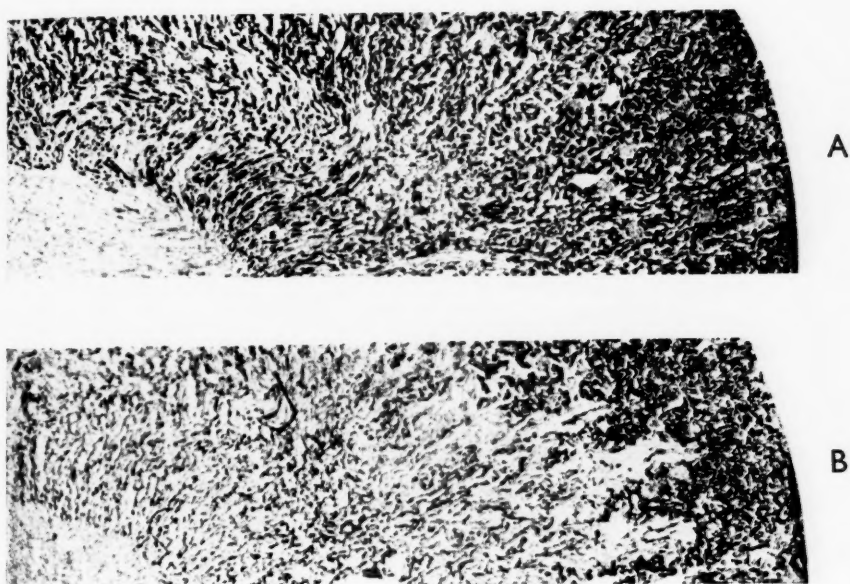


Fig. 2. — Histochemistry of ICD
A Control (incubation 45 min)
B After Esidron, 20 mg/kg.

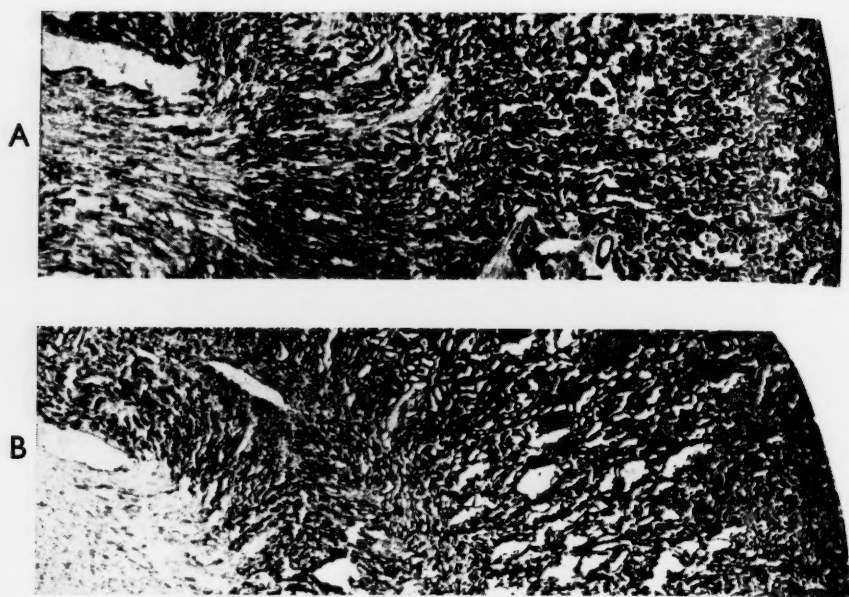


Fig. 3.— Histochemistry of LDH

A Control (incubation 45 min)

B After mercaptomerin, 30 mg/kg.

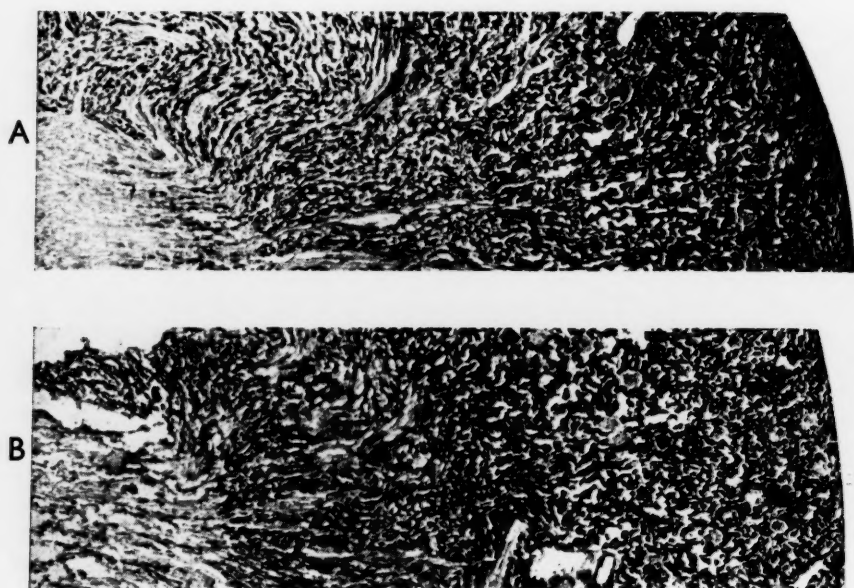
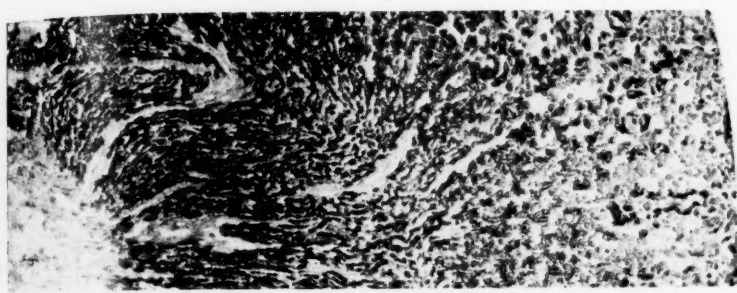


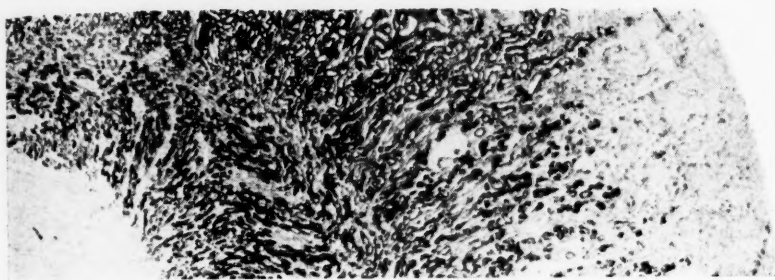
Fig. 4. — Histochemistry of MDH

A Control (incubation 45 min)

B After mercurphylline, 30 mg/kg.

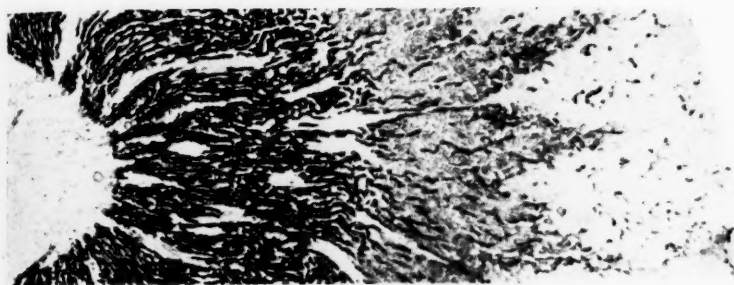


A

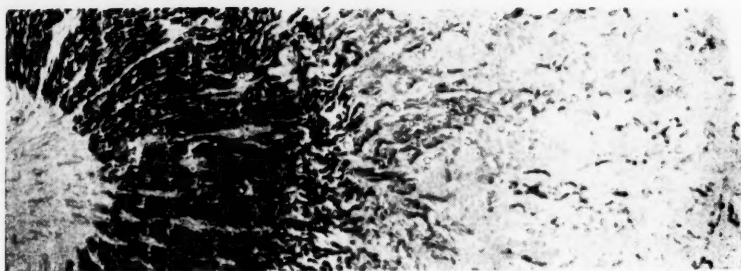


B

Fig. 5. — Histochemistry of α -GPDH
A Control (incubation 60 min)
B After mercaptomerin, 30 mg/kg.



A



B

Fig. 6. — Histochemistry of β -HBDH
A Control (incubation 60 min)
B After Esidron, 20 mg/kg.

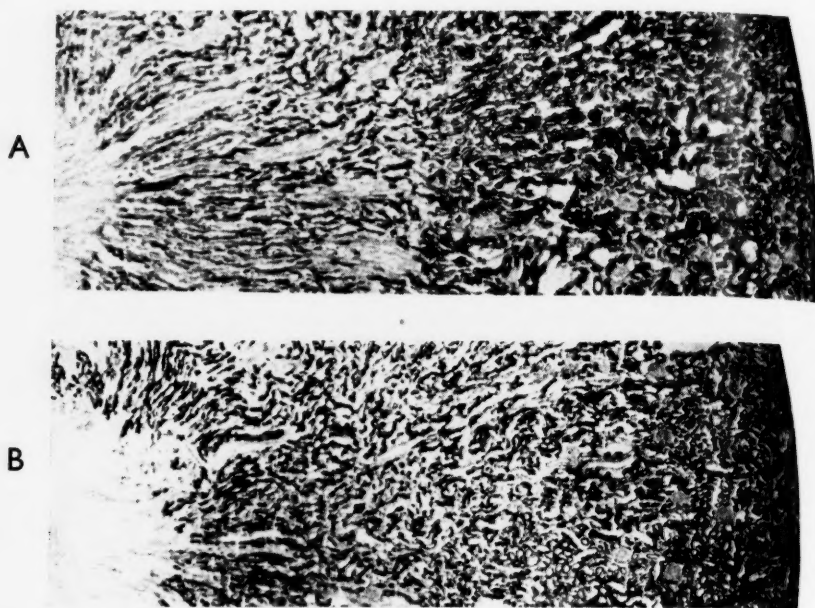


Fig. 7. — Histochemistry of GLUT DH
A Control (incubation 60 min)
B After meralluride, 20 mg/kg.

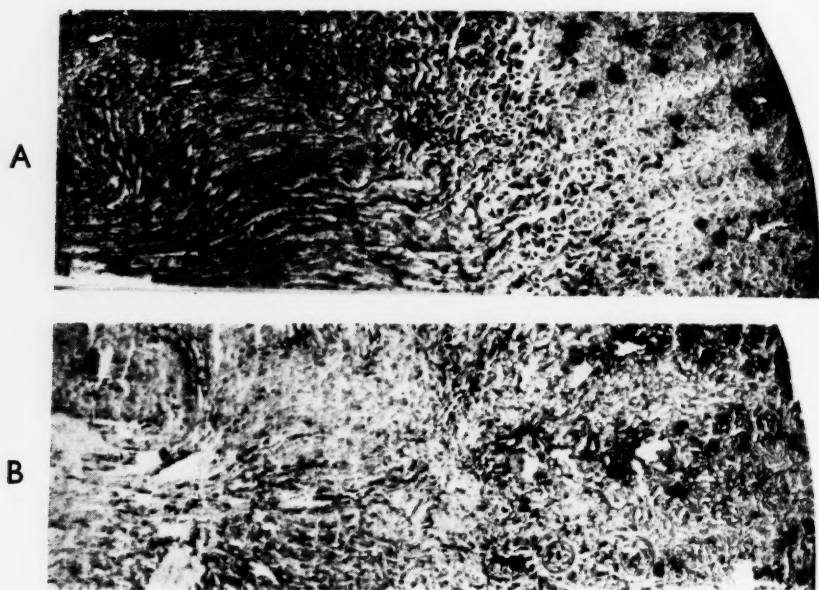


Fig. 8. — Histochemistry of ATP-ase
A Control (incubation 30 min)
B After meralluride, 20 mg/kg.

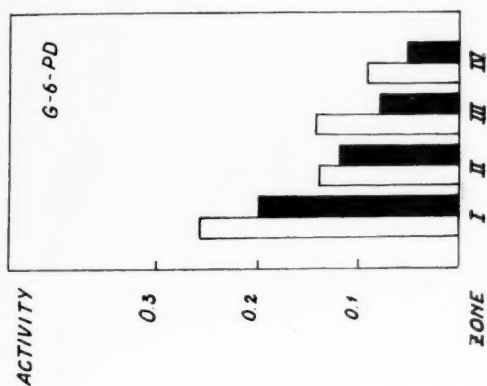


Fig. 9.

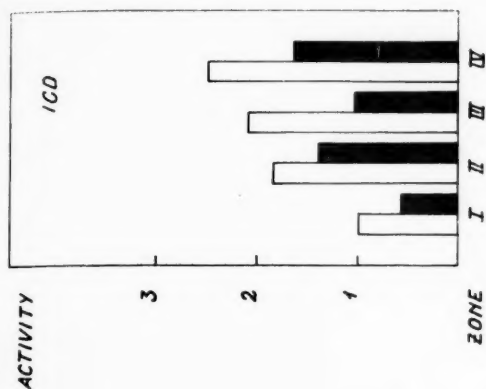


Fig. 10.

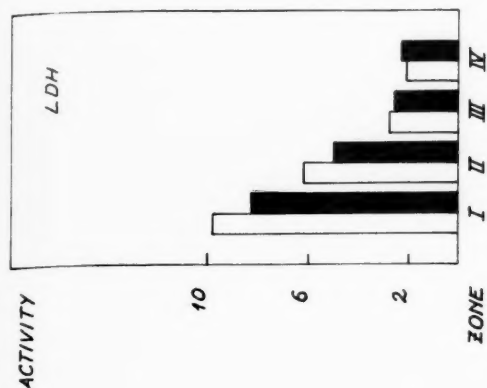


Fig. 11.

Fig. 9—23. Mean activities of 12 enzymes (for abbreviations see footnote on page 188) in homogenates of four macroscopically distinguishable renal zones of normal (white columns) and mercurial diuretic-treated (black columns) rats. The units for G-6-PD, ICD, LDH, MDH, GOT, GR and ALD are Δ O.D. $\times 10^{-3}$ /minute/ μ g of tyrosine, for β -GLUC μ g phenolphthalein liberated in 30 minutes/ μ g of tyrosine, for ISOM μ M of fructose-6-phosphate formed/minute/ μ g of tyrosine, and for the phosphatases μ g of P released in 30 minutes/ μ g of tyrosine. Zones: I papilla, II outer medulla, III inner medulla, IV outer cortex.

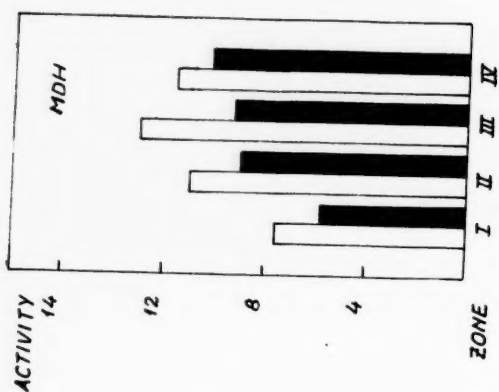


Fig. 12.

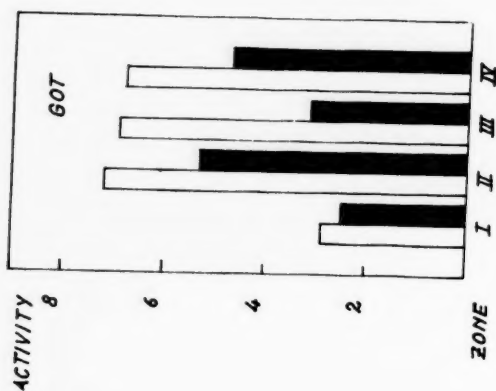


Fig. 13.

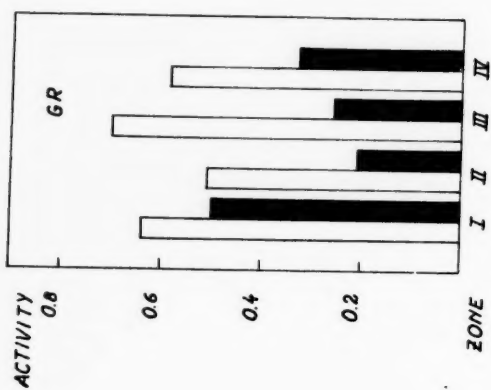


Fig. 14.

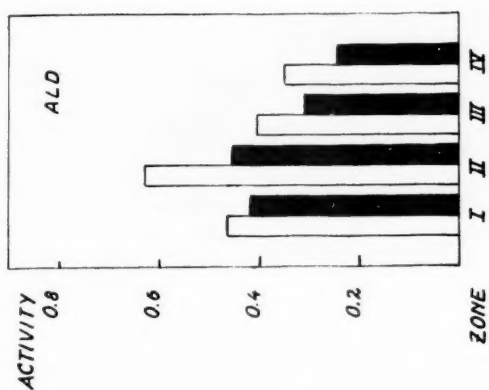


Fig. 17.

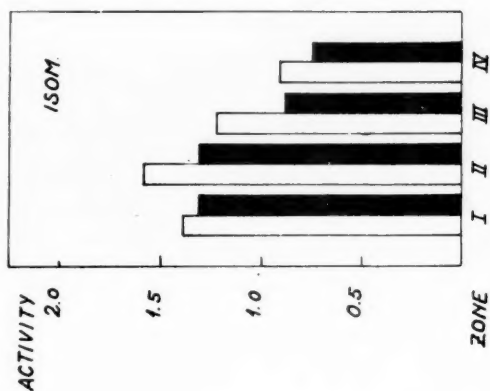


Fig. 16.

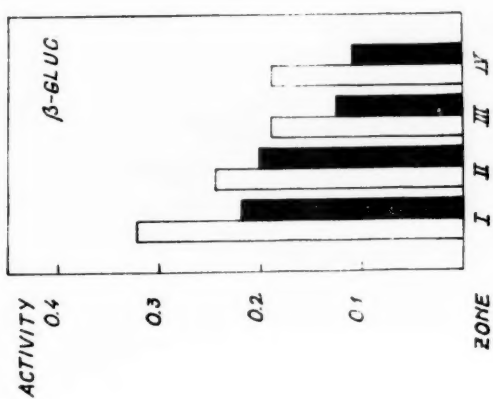


Fig. 15.

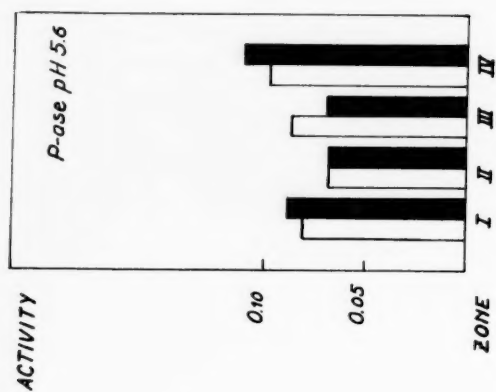


Fig. 18.

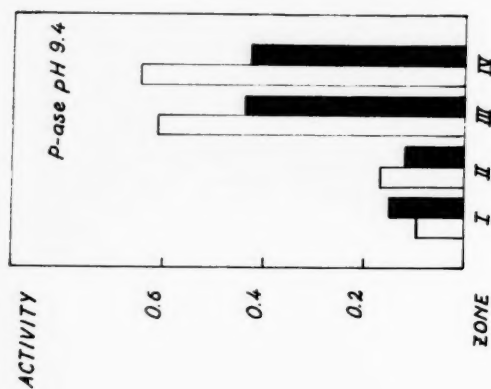


Fig. 19.

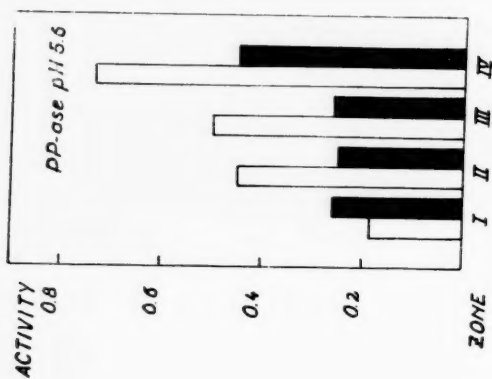


Fig. 20.

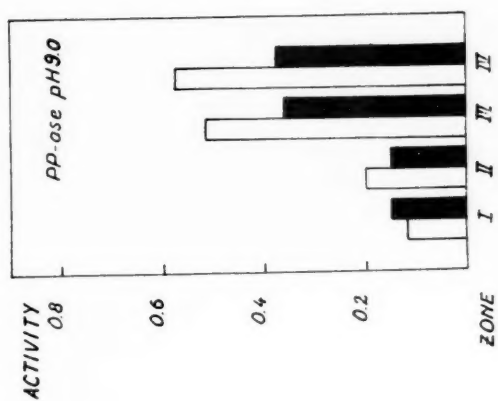


Fig. 21.

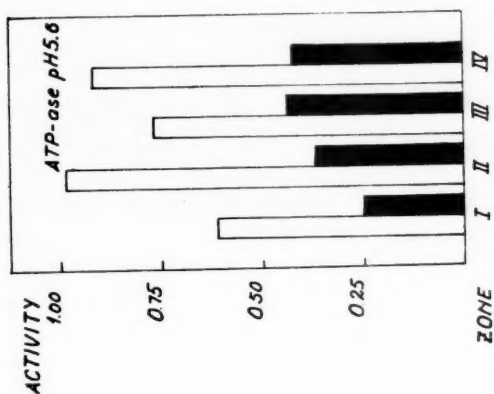


Fig. 22.

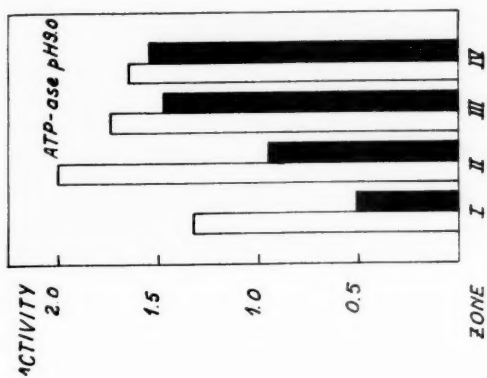


Fig. 23.

sections incubated with Nitro-Blue Tetrazolium, no inhibition could be detected in the ascending thick limb of Henle's loop.

Chemical Assays. — The activities of 12 enzymes determined in homogenates of the four kidney zones and referred to soluble protein (tyrosine) content are presented in figures 9 through 23. White columns indicate the mean activity in untreated control animals and the black ones stand for the mean of all mercurial diuretic-treated rats irrespective of the preparation and dose given. This manner of illustration is justified because the four mercurials studied were about equally effective when referred to mercury content.

In accordance with the histochemical observations the inhibitory action of mercurial diuretics appeared, in general, to be greatest in zone III (inner cortex). Of the four dehydrogenases studied the TPN-dependent ones (G-6-PD and ICD) were significantly inhibited whereas the DPN-linked LDH and MDH remained unaffected. The GOT activity was also decreased in zone III, the GR was markedly inhibited in zones II through IV. Definite effects were produced on different phosphatases. The ATP-ase was inhibited to a considerable extent in all zones at pH 5.6 and in zones I and II at pH 9.0 exhibiting thus a unique pattern. Pyrophosphatase and alkaline glycerophosphatase activities were somewhat decreased also in zones I and II. Acid glycerophosphatase, ISOM, ALD, β -GLUC activities were slightly or not at all inhibited by the diuretics. Omission of cysteine did not significantly alter the results.

Acetazolamide and dihydrochlorothiazide were without effect on the enzymes studied both by histochemical and chemical means.

DISCUSSION

The unipapillary kidney of rat is particularly suited for macrodissection, since it can be easily divided into four zones containing definite segments of the nephron. Histochemical studies on several renal enzymes have revealed a clearcut zonal distribution (21), but a quantitative enzyme pattern has so far been determined only for succinic dehydrogenase (11). In the present study the quantitative principle has been extended to comprise 12 enzymes, assayed in homogenates of dissected zones. The results were reasonably parallel to those obtained by histochemical methods (as regards the five enzymes, which were determined by both methods).

The histochemical method, though so far definitely superior to the chemical *in vitro* assay in the exact localization of enzymes, suffers from the drawback of being only arbitrarily quantitative. This fact is of particular importance in attempts to detect small enzyme activations and inhibitions as they occur in physiological control of metabolism and often also due to pharmacological agents. On the other hand, the spatial dearrangement due to the loss of the cellular structure on homogenization and the otherwise rather unnatural milieu under which the enzyme acts in the *in vitro* assay system are inherent drawbacks of the quantitative method. Though the dilution of enzyme and inhibitor in the homogenate is equal, their effects need not necessarily be »diluted» proportionately. In addition, in the whole tissue homogenate the activity of uninhibited structures may partially obscure small inhibitions. This is a probable explanation for the finding that, in regard to dehydrogenases at least, the quantitative assay seems not to be a more sensitive indicator of mercurial inhibition than the histochemical method.

That nephrotoxic doses of mercurials are to be administered in order to produce the enzyme inhibitions reported, has been criticized by several investigators (4, 13). Rat is, however, known to require high doses of mercurials for enhancement of diuresis (6). Moreover, only an one per cent inhibition in the tubular reabsorptive capacity is needed to an effective diuretic response. Such an inhibition is not measurable by the current techniques of enzymology. To avoid nephrotoxic single doses of mercurials, Hess (9) administered small doses over prolonged periods. This manner can be criticized, however, because the possibility of enzyme adaptation cannot be excluded. The localization of mercurial action in the nephron seems not to be invalidated by the high doses used, since it has been generally assumed that therapeutic doses of mercurials act at the same sites (22). Little can be said of the significance of the observed enzyme inhibitions in the diuretic action, but they are obviously not mere manifestations of necrobiotic changes. This claim is substantiated by the finding that the strength of the inhibition roughly paralleled the sensitivity of individual enzymes to sulfhydryl blocking agents (3).

SUMMARY

The effect of mercurial diuretics, acetazolamide, and dihydrochlorothiazide on the enzyme pattern of rat kidney has been studied both by histochemical techniques and by a quantitative *in vitro* assay in homogenates of four separated renal zones.

The localization of the following enzymes was carried out histochemically: glucose-6-phosphate dehydrogenase, isocitric dehydrogenase, lactic dehydrogenase, malic dehydrogenase, β -hydroxybutyric dehydrogenase, α -glycerophosphate dehydrogenase, glutamic dehydrogenase, succinic dehydrogenase, and ATP-ase. The following enzymes were determined quantitatively: glucose-6-phosphate dehydrogenase, isocitric dehydrogenase, lactic dehydrogenase, malic dehydrogenase, glutamic oxalacetic transaminase, glutathione reductase, phospho hexose isomerase, diphosphofructaldolase, β -glucuronidase, glycerophosphatase, pyrophosphatase, and ATP-ase, each of the three phosphatases at two pH-values.

The results obtained by the histochemical and by the quantitative methods were similar. Corresponding to the histochemical localization of mercurial action to the terminal portion of proximal convoluted tubules, the inhibition of enzymes was most pronounced in Zone III containing these tubular segments. The strength of the inhibition roughly paralleled the sensitivity of the individual enzymes to sulfhydryl blocking agents. No effects were observed after administration of acetazolamide and dihydrochlorothiazide.

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The authors are obliged to Kaarina Ojala, M.Sci. for skillful assistance in this work.

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THE URINARY EXCRETION OF RADIOACTIVE MATERIAL
AFTER ADMINISTRATION OF C¹⁴-LABELLED MENADIONE (2-METHYL-
C¹⁴-1,4-NAPHTHOQUINONE)*

by

B.-A. LAMBERG and R. GORDIN

(Received for publication January 27, 1960)

C¹⁴-labelled menadione (2-methyl-C¹⁴-1,4-naphthoquinone) was synthesized in 1952 by Phillips, Trevoy, Jaques and Spinks (9). Later on, the same group of investigators carried out various experiments concerning the distribution and excretion of labelled menadione in experimental animals (4, 5, 10). In the mouse the urinary excretion was extremely rapid, most of the material being excreted during the first few hours after intramuscular injection. Only about 50 per cent of the dose was recoverable, however, about 50 per cent disappearing in some unknown manner. No storage of menadione in the organs of the mouse could be demonstrated.

It thus seemed to be of interest to study the excretion of C¹⁴-labelled menadione in man, especially in patients with disturbances of prothrombin synthesis.

METHODS

C¹⁴-labelled menadione with a specific activity of 1.44 mc/mM (equal to 500 μ c/59.7 mg) (Amersham, England) was dissolved in olive oil and administered orally to patients in doses of 14.7 to 17.2 μ c (1.75 to 2.05 mg) in a gelatine capsule. Urine was collected at specified intervals. The volume was reduced to 100 ml by slow

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drying. Dry preparation of «infinite» thickness were made up from 2 ml samples in aluminium planchets and the radioactivity was counted with a thin-window Geiger-Müller tube. In one experiment standard preparations were made up in the same way, using a water-soluble C^{14} -labelled substance.

Two series of experiments were made. In the first, menadione was administered to 6 patients, 3 cases of toxic nodular goitre and three control subjects without evidence of liver, heart or kidney disease. In the hyperthyroid patients the serum prothrombin determined according to the P-P method of Owren and Aas (8) was below normal, varying from 50 to 70 per cent. In one of the control subjects the prothrombin level was slightly decreased (60—80), probably owing to an acute infectious condition. The urine was collected at 8, 16, 24, 36, 48, 60 and 72 hours after administration of the vitamin. The radioactivity, expressed as counts per hour in the 2 ml sample measured, was divided by the number of hours of the corresponding collection period. Hence, the slope of the curve in fig. 1 should correspond to the slope of a curve depicting the excretion rate.

In the second experiment the vitamin was given to 6 patients, — 3 cases with toxic nodular goitre, one of which had a decreased prothrombin level (44%), 1 with infectious jaundice, 1 with syphilitic cirrhosis of the liver and 1 control subject. The urine was collected at 4, 8, 16, 24, 36, 48 and 72 hours after administration of the dose. The counting rate of the urinary sample measured was compared with the standard preparation containing a known amount of C^{14} . The results were expressed as per cent of the dose.

In two of the cases, the one with syphilitic cirrhosis and the control subject, a dilution test was carried out. After 72 hours from the start of the experiment 80 mg of non-labelled menadione was given intramuscularly and the excretion of radioactive material was measured for a few more days. All of the patients studied except 3 were above 50, most of them above 60 years of age.

RESULTS

The results of the first experiment are shown in fig. 1. The half-time ($T_{1/2}$) of the excretion curves varied from about 2 to 4 hours during the first 16 hours, after which the slope changed

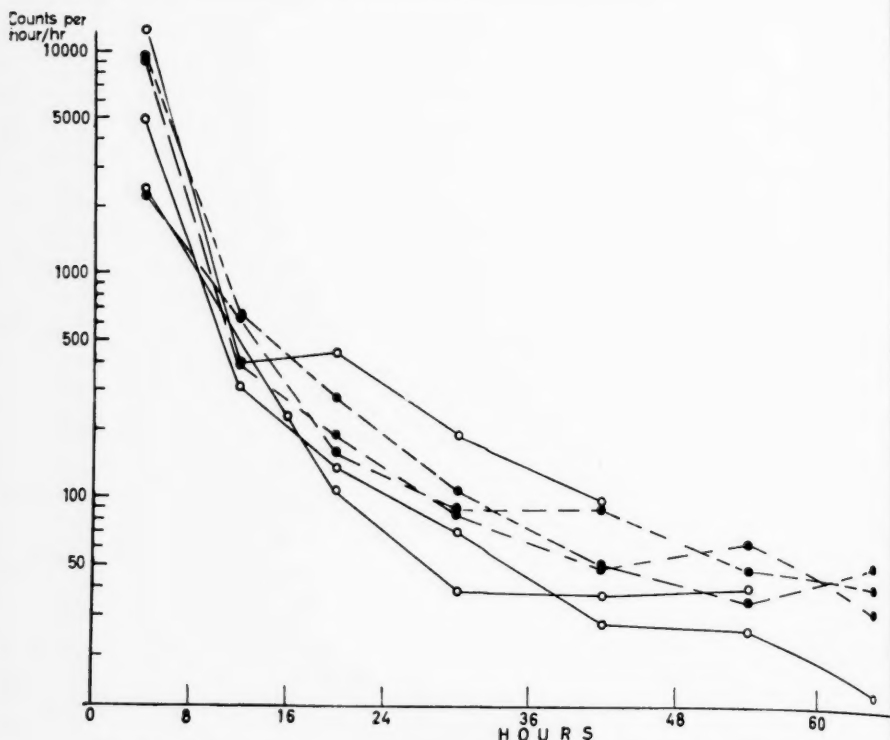


Fig. 1. — The excretion of radioactive material in the urine after ingestion of menadione- C^{14} . For explanation see text.

○ — hyperthyroid patients
● — control subjects

gradually, $T_{1/2}$ being more prolonged. During the next 24 hours the $T_{1/2}$ was about 10–12 hours.

The results of the second experiment are depicted in figs 2 and 3. The cumulative excretion curve in fig. 2 shows that most of the radioactivity was excreted during the first 24 hours.

The largest portion was excreted during the first 8 hours. After 24 hours 37.6 to 68.8 per cent of the dose had been excreted and from this time on the daily excretion varied from 0.158 to 0.434 per cent. Hence, the cumulative excretion curve attained nearly a plateau from 24 hours on.

In the same figure the result of the dilution test is shown. The administration of 80 mg of menadione intramuscularly did not increase the rate of excretion of radioactive material.

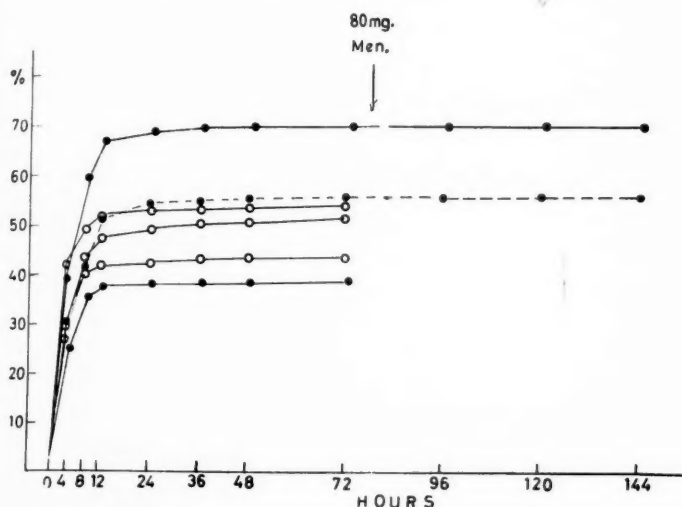


Fig. 2. — The cumulative excretion of radioactive material in the urine after ingestion of menadione- C^{14} . No effect on the excretion was observed in the dilution test when 80 mg non-labelled menadione was administered intramuscularly.

- — — — ● hyperthyroid patients
- — — — ○ patients with liver damage
- — — — ● control subject

The excretion rate expressed as per cent of the dose per hour is depicted in fig. 3. It varied from 6.75 to 10.3 per cent per hour during the first 4 hours. The $T_{1/2}$ of the curve during the first 12 hours varied from about 2 to 4 hours, indicating an excretion rate of about 17 to 35 per cent of the menadione pool per hour. From 12 hours on, the $T_{1/2}$ was about 6 to 8 hours for some time, being even more prolonged later on.

In several patients the urine was collected also during the period 72–144 hours during which a constant daily excretion ranging from about 0.3 to 1.0 per cent of the retained radioactivity was observed. This is well in agreement with a final state of equilibrium and also with the findings of Solvonuk *et al.* (10) according to which no storage could be found. On the basis of these excretion data the effective $T_{1/2}$ would range approximately from 70 to 200 days corresponding to a total radiation dose of 7 to 20 rem at the outmost provided that the urinary route would be the only route of excretion which is improbable.

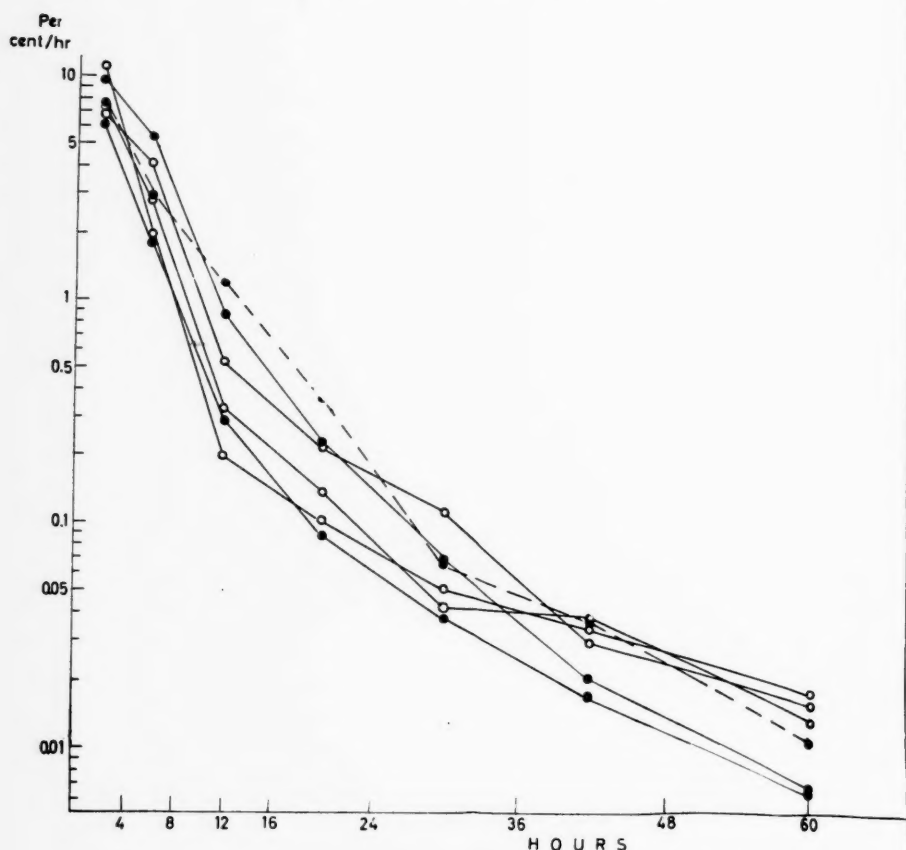


Fig. 3. — The urinary excretion rate of radioactive material after ingestion of menadione- C^{14} . Symbols as in fig. 2.

No evident correlation was observed in any experiment between the excretion rate and the diagnosis or the prothrombin level.

DISCUSSION

The present experiments indicate that the urinary excretion of menadione is also very rapid in man. The shape of the cumulative excretion curves was very similar to that reported for the mouse by Solvonuk *et al.* (10). In the present experiments most of the radioactive material was excreted during 24 hours, the bulk being excreted during the first 8 hours. After 24 hours the daily

excretion varied from 0.158 to 0.434. After 72 hours the total excretion was 55.4 per cent in the control subject, 38.4 per cent in a case with infectious jaundice, 69.8 per cent in a case with syphilitic cirrhosis of the liver and 43.7, 51.0 and 53.8 per cent resp. in the three patients with toxic nodular goitre. On the basis of this small series no definite conclusion can be drawn as regards the total excretion in various diseases. No clear differences from the control subject were noted, however, in the excretion rate in the cases with hyperthyroidism or liver disease.

The inclusion of hyperthyroid patients in this study was prompted by the observations previously reported (2, 6) that the prothrombin level was decreased in about 50 per cent of these cases.

The total excretion within the first few days was on an average slightly higher than that reported for the mouse (5, 10) and for the rat (1) in which the maximal excretion was 30 and 20 per cent respectively. According to the experiments of Solvonuk *et al.* (10) only about 50 per cent of the menadione administered was recoverable, the rest disappearing in some unknown manner. This finding was thought possibly to be due to dilution, the radioactivity in various parts of the body thus not being measurable. No storage could be demonstrated in any tissue.

Vitamin K_1 , however, behaves differently. In contrast to menadione, vitamin K_1 is recoverable to nearly 100 per cent, a large proportion being found in the intestine (5) and some in the liver (11, 12). Even after the application of a bile fistula menadione is not found in the faeces, whereas vitamin K_1 is present to about 100 per cent (5).

In the present experiments the dilution tests did not increase the urinary excretion, indicating that the menadione in the body is not present in a readily exchangeable pool of unchanged substance. It would be of interest to repeat the experiments with the labelled vitamin K_1 which has been synthesized by the Canadian group of investigators (7, 13) in animals.

The shape of the curve of the excretion rate in the present experiments indicates that more than one rate constant is involved. The reason for this may only be speculated upon as neither the experiments of Solvonuk *et al.* (10) nor the present findings furnish any definite clues. One process most probably accounting for the

existence of another rate constant seems to be the formation of metabolites of menadione. Hoskin (3) and Hoskin, Spinks and Jaques (4) have demonstrated that menadione is not excreted in the urine in unchanged form but partly as diglucuronide and partly as monosulphate. Furthermore, they also found an unidentified product in the urine. The formation of these metabolites and possibly the different behaviour of these metabolites with regard to glomerular filtration rate and tubular reabsorption may result in different rate constants influencing the shape of the excretion curve of radioactive substances.

SUMMARY

The urinary excretion of radioactive substances was studied in patients with various conditions after oral administration of C^{14} -labelled menadione (2-methyl-1,4-naphthoquinone). The excretion was very rapid, most of the radioactive material being excreted during the first 24 hours. After 72 hours the total excretion varied from 35 to 70 per cent. The shape of the curve for the excretion rate expressed as per cent per hour suggests the presence of several rate constants influencing the excretion curve.

Acknowledgements. — The writers wish to express their gratitude to Mrs S. Jokinen for technical assistance.

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ABO BLOOD GROUPS IN PERNICIOUS ANAEMIA AND PERNICIOUS TAPEWORM ANAEMIA

by

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(Received for publication January 19, 1960)

Statistics have been prepared recently on the distribution of blood groups in certain diseases. The data on pernicious anaemia suggested that group A is relatively larger in this disease than in the control materials (1, 2, 3, 4). The statistics in question are small. In order to supplement the picture, we have collected some figures from Finland and, in addition, some concerning the tapeworm anaemia. For 138 pernicious anaemia and 197 tapeworm anaemia cases the percentual distribution into ABO blood groups is shown in Table 1.

In pernicious anaemia the incidence of the group O was smaller than in the control series and smallest in the tapeworm anaemia.

TABLE 1

DISTRIBUTION OF BLOOD GROUPS IN FINLAND IN PERNICIOUS ANAEMIA AND PERNICIOUS TAPEWORM ANAEMIA. CONTROL GROUP COLLECTED BY O. STRENG (6)

	Number of Patients Examined	Percentual Distribution of Blood Groups			
		A	O	B	AB
Cryptogenetic pernicious anaemia ..	138	43.4	29.0	16.7	10.9
Pernicious tapeworm anaemia ..	197	43.6	24.9	19.3	12.2
Control series (6) ..	29915	42.3	32.9	17.9	6.9

If the A/O ratio is calculated by means of the formula $\frac{Ap \times Ok}{Op \times Ak} = I$, the index in pernicious anaemia in the above materials ranges from 0.98 to 1.70 (5). In the present authors' material the comparable figure is 1.17 in pernicious anaemia and 1.37 in tapeworm anaemia.

The authors consider that the determination of ABO groups in cases of tapeworm anaemia may be of value in assessing the pathogenesis of the disease, especially as this disease is known not to occur in all worm carriers.

SUMMARY

The distribution of ABO groups in 138 cases of pernicious anaemia and 197 cases of tapeworm anaemia in Finland was studied. In agreement with the picture given by previous investigations, the A/O ratio was greater in pernicious anaemia than in the control series. It was particularly noteworthy that this ratio was even greater in pernicious tapeworm anaemia.

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THE EFFECT OF PHENOBARBITAL ON THE LIVER

by

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The metabolism of several barbiturates is considered to occur principally in the liver tissue, and in fact normal liver function is regarded as important for the detoxication of barbiturates (2). On the other hand, barbiturates have also been reported as impairing liver function, especially in large doses. In barbiturate poisoning, for instance, some patients develop jaundice and the microscopic picture of the disease resembles fatty degeneration of liver (3) or acute liver atrophy (6). Administered in therapeutic doses, however, barbiturates do not seem to impair liver function. An anaesthetic dose of amobarbital causes no decrease in bromsulphalein excretion either in rabbit or in man (1). Even frequently repeated anaesthetic doses of thiopental disturb dog liver function only slightly (7).

In an earlier investigation in which repeated injections of non-anaesthetic doses of phenobarbital were given to rats, our attention was drawn to the enlargement of the liver during the treatment. The phenomenon had not, as far as could be ascertained, been reported in the literature. This and especially the fact that the detoxication and elimination of phenobarbital take place chiefly via the kidneys (2) prompted us to make a further study of the problem.

The rats were given repeated phenobarbital doses twice a day and the changes in the liver were examined at various intervals.

MATERIAL AND METHODS

Groups of white rats weighing 160—220 g at the beginning of the test were used. The animals were kept in uniform test conditions, and on a standard diet.

Phenobarbital (»Barbiphenyl», generously supplied by Orion Oy, Helsinki), 50 mg/kg, was injected subcutaneously into the animals twice a day. The control animals were given the mixture used to dissolve the phenobarbital, also twice a day subcutaneously. The test animals were killed after 1, 3, 5, 8 and 14 days of injections, and after 14 days of injections followed by a 1-week and a 2-week period without any treatment. The object of the last-mentioned series was to ascertain the permanence of the changes provoked by phenobarbital. The test animals and their livers were weighed and samples were taken to establish the dry weight of the liver and for microscopic examination.

The dry weight of liver was determined by placing a weighed quantity of liver tissue in a desiccator containing concentrated sulphuric acid; the pieces of liver thus dried were weighed once a week. When the weight was constant on two successive occasions the last two readings were taken as the weight of the dry substance of the liver.

Microscopic examination was made of preparations treated in the following way: pieces of the liver were fixed immediately after sacrifice and weighing in 10% formaldehyde and Schaffer's (2 parts of 80% alcohol + 1 part of 40% formaldehyde) alcohol-formol. Frozen sections were cut from the formol-fixed preparations and stained with Scharlach R according to Michaelis for examination for fat.

Normal paraffin block was made of the Schaffer-fixed preparations which were stained with Best's carmine for glycogen examination. For the sake of control, one staining was made in each group according to v. Gieson.

RESULTS

The weight of the liver in the group receiving phenobarbital (Table 1) increased steadily from the first day. The difference from the control group was statistically significant from the 1st day on, and after that, highly significant. After the 2-week course of

TABLE 1
THE EFFECT OF PHENOBARBITAL INJECTIONS ON THE LIVER WEIGHT AND THE DRY SUBSTANCE
OF THE LIVER

	Control	Injection Period				
		1 Day	3 Days	5 Days	8 Days	14 Days
Weight of liver, per cent of body weight, mean and standard deviation (Number of animals)	3.95 ± 0.16 (n 15)	4.60 ± 0.15 (n 3)	5.06 ± 0.20 (n 5)	5.81 ± 0.46 (n 7)	6.70 ± 0.06 (n 3)	6.53 ± 0.3 (n 6)
Dry substance, per cent of the fresh weight	31.8	33.7	31.0	33.3	29.7	32.8

Significance of the difference between the groups:

Liver weight:

Control — 1 day 0.01 > P > 0.001

“ 3 days P < 0.001

“ 5 days P < 0.001

“ 8 days P < 0.001

“ 14 days P < 0.001

Dry substance: no significant differences.

TABLE 2
ELIMINATION OF THE LIVER WEIGHT INCREASE CAUSED BY PHENOBARBITAL INJECTION

	Control	After Last Injection	
		7 Days	14 Days
Weight of liver, per cent of body weight, mean and standard deviation (Number of animals)	3.95 ± 0.16 (n 15)	7.63 ± 0.65 (n 3)	4.37 ± 0.14 (n 1)
Dry substance, per cent of the fresh weight	31.8	30.0	35.3

Significance of the difference between the groups:

Liver weight:

Control— 7 days without phenobarbital P < 0.001

“ 14 “ “ “ “ P > 0.05

Dry substance: no significant differences.

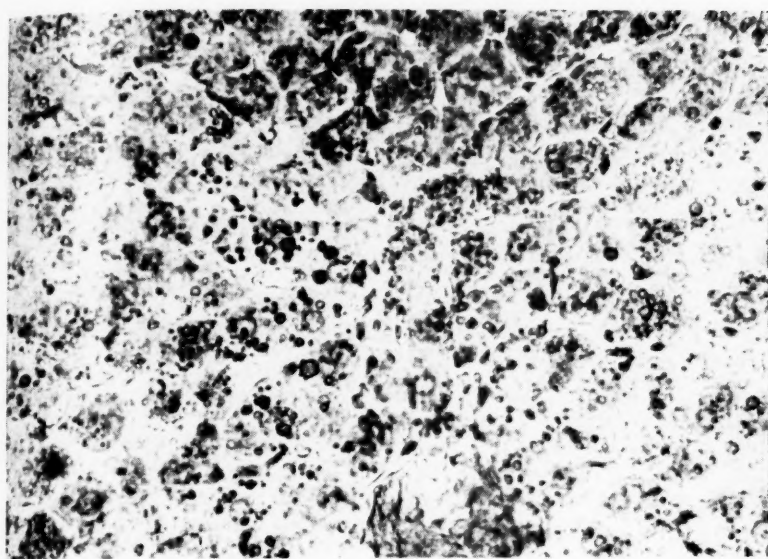


Fig. 1. — Fat deposits in the parenchymal cells of rat liver after 5 days' phenobarbital therapy. The fat appears as black globules and rings. Microphoto $\times 300$. Frozen section Scharlach R.

injections the liver of the test animals weighed nearly twice that of the controls. When the injections were discontinued, the difference seemed to disappear in 2 weeks (Table 2). The fat deposits in the organs, especially the perirenal fatty tissue, also diminished in several tests.

The weight of the rats given phenobarbital seemed to develop similarly to that of the control animals during the test. Three of the phenobarbital animals died during the test.

The groups showed no statistically significant difference in the dry weight proportion of the fresh liver weight.

Macroscopically, the liver of the rats receiving phenobarbital was more yellowish and the lobular structure was less distinct than in the controls. No difference was established microscopically in the glycogen content of the different groups. Fat staining, on the other hand, revealed a considerable increase in fat in the animals given phenobarbital. In most of the preparations, the fat was distributed evenly in small globules in the parenchymal liver cells. Animals on the long-term phenobarbital regimen, however, showed a greater density of fat drops in the centre of the lobule, suggestive of typical central fatty degeneration (Fig. 1).

DISCUSSION

The liver of the animals receiving phenobarbital was clearly enlarged during the treatment. The process begins on the first day, the enlargement is considerable in 1—2 weeks, and it disappears in about 2 weeks after the discontinuance of phenobarbital administration. The fact that the dry weight of the liver tissue in per cent from the fresh weight remained unchanged in both the animals which received phenobarbital and in the control animals suggests that the weight increase is not caused by volatile substances, such as water. It may thus be possible in searching for an explanation of the enlargement of liver to exclude the accumulation of water which may result from circulatory damage possibly caused by phenobarbital. Microscopic examination showed that the fat content of the liver increased. The weight gain thus seems to be attributed at least to a great extent to the fat deposited in the liver.

The phenomenon is interesting especially because the phenobarbital dosage was less than half of the anaesthetic dose (5). Barbiturates have previously been found to cause an increase in the phosphatase content of the liver (4). It cannot be said whether the present finding of fat accumulation in the liver is associated with the increase in phosphatases. More detailed investigations on the factors associated with the accumulation of fat in the liver are in progress.

SUMMARY

Rats injected subcutaneously with 50 mg/kg of phenobarbital twice a day showed a considerable increase in the weight of the liver from the first day. The weight per weight unit of the body had almost doubled after 2 weeks. The dry substance of the liver in per cent of the fresh weight remained the same as in the control animals, and microscopic examination showed that the fat in the liver had increased considerably. The increase in the weight of the liver disappeared within 2 weeks after the discontinuance of the injections.

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THE EFFECT OF RESERPINE AND NEOSTIGMINE ON THE EXCRETION OF 5-HYDROXYTRYPTAMINE AND 5-HYDROXYINDOLEACETIC ACID IN RABBITS

by

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(Received for publication January 29, 1960)

The greatest amount of 5-hydroxytryptamine (5HT) in mammalian tissues is generally present in the gastro-intestinal tract, where it is probably located in the enterochromaffin cells (8). Rauwolfia alkaloids, such as reserpine, cause a marked decrease in the 5HT content in various tissues, including the intestine (18). At the same time they increase the excretion of 5-hydroxyindoleacetic acid (5HIAA) (23), considered the main metabolite of 5HT in most animals.

5HT stimulates intestinal and other smooth muscle preparations. It has been demonstrated on guinea pigs that 5HT release from the intestinal mucosa into the lumen is increased when intraluminal pressure is elevated (3). Reserpine increases the motility of the intestinal tract *in vivo* and a probable explanation for this is the 5HT set free from its binding sites. The stimulation of the intestine by other means has not lead to any measurable depletion of 5HT stores (18).

The intestinal 5HT content is more resistant to Rauwolfia treatment than the 5HT content of other tissues (4, 7, 14). We can assume that during the reserpine action part of the 5HT still present in the intestine is more loosely bound than normally in the cells. Under these circumstances one might expect that an additional increase in motility would increase the rate of 5HT release and 5HIAA excretion even though the stimulant when given alone,

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would be ineffective in this regard. We have studied whether the anticholinesterase neostigmine alone has an effect or whether it increases the influence of reserpine on the 5HT and 5HIAA excretion and the 5HT content in the intestine.

METHODS

Rabbits of both sexes, weighing from 1.9 to 3.2 kg were used. They were allowed to eat hay, swedes and oats until the experiment was started. The abdomen was opened under ether anesthesia. To collect urine into the bladder the urethra was closed after emptying the bladder by compressing it. At the same time an artificial ileus was produced by closing the intestine just above the ileocecal junction. After closing the wound, the animals were left to recover and then were killed four hours after the operation by a blow on the head and the thorax was opened.

The following drugs were used: reserpine (Lääke Oy, Turku) (dissolved in glacial acetic acid and diluted with water) 1.5 mg (1 ml)/kg intravenously; neostigmine methylsulphate (A.B. Leo, Hälsingborg) 0.2 mg/kg subcutaneously in three divided doses. Reserpine and the first dose of neostigmine were injected after completing the operation.

A piece of about one gram of small intestine (approximately 30 cm above the ligature) was taken for 5HT estimation. The extraction was done with acetone and heptane, as previously described (16). The blood was withdrawn from the left ventricle. 95% acetone (final concentration) was used for the extraction of the blood and urine (1). Both blood and urine samples were washed once with petroleum ether. 5HT was assayed biologically by using the rat stomach method (25). The doses refer to the base.

The estimation of 5HIAA was made by the spectrophotometric method (24) (Beckman spectrophotometer DU). Treatment with dinitrophenyl-hydrazine and chloroform extraction were omitted, because there was no interference from ketoacids. The amount of 3-indoleacetic acid was less than 5 μ g/ml of urine. For the identification of 5HIAA and the estimation of 3-indoleacetic acid in several samples the following procedure was used: Acidified rabbit urine was first extracted with ether to eliminate tryptophan. After using paper chromatography (Whatman No 4 paper) in solvent containing

NaCl: acetic acid: water (8: 1: 100) (13) the spots were developed by 1-nitroso-2-naphthol. The intensity of 5HIAA spot was comparable to the amount found by the spectrophotometric method. The area corresponding the Rf of 5HIAA was first extracted in ether and then in phosphate buffer. The absorption spectrum of the chromophor produced by nitrosonaphthol was similar to that of synthetic 5HIAA.

RESULTS

The results are given in Table 1. It is probable that reserpine induced a decrease of 5HT in the intestine of the rabbits although this was not significant because of the large range of the values. Neostigmine was apparently inactive in this respect. Neither did it produce any measurable increase in the action of reserpine in lowering the 5HT stores in this organ. The lumen of the intestine

TABLE 1

THE EFFECT OF RESERPINE (1.5 MG/KG *i.v.*) AND NEOSTIGMINE (0.2 MG/KG *s.c.* IN DIVIDED DOSES) ON THE 5-HYDROXYTRYPTAMINE CONTENT OF THE INTESTINE, WHOLE BLOOD AND URINE AND ON THE 5-HYDROXYINDOLEACETIC ACID (5HIAA) IN THE URINE OF RABBITS. EXPERIMENTAL INTESTINAL OCCLUSION WAS MADE IN ALL GROUPS EXCEPT THE LAST. THE MEANS \pm S.E. OF THE MEAN ARE GIVEN. ALL THE RESULTS HAVE BEEN COMPARED WITH THE CONTROL GROUP VALUES BY USING STUDENT'S T-TEST AND P-VALUES ARE GIVEN ONLY WHEN THERE IS A SIGNIFICANT ($p < 0.05$) DIFFERENCE. FOR FURTHER DETAILS, SEE METHODS

Treatment (and Number of Animals)*	5-Hydroxytryptamine			5HIAA
	Intestine $\mu\text{g/g}$	Blood $\mu\text{g/ml}$	Urine $\mu\text{g/hr}$	Urine $\mu\text{g/hr}$
Controls (6)	6.12 ± 1.66	1.31 ± 0.24	1.72 ± 0.28	26.8 ± 7.10
Reserpine (8)	4.24 ± 1.26	1.16 ± 0.24	$2.85(7) \pm 0.34$ $p < 0.05$	146 ± 15.9 $p < 0.001$
Neostigmine (8)	6.14 ± 0.92	1.22 ± 0.31	$0.96(7) \pm 0.15$ $p < 0.05$	22.4 ± 5.09
Reserpine + Neostigmine (8)**	3.76 ± 0.57	0.73 ± 0.20	3.66 ± 0.29 $p < 0.001$	$84.6 \pm 17.4^{**}$ $p < 0.01$
No Intestinal Occlusion (5)	4.78 ± 1.29	1.78 ± 0.28	1.86 ± 0.32	14.0 ± 4.00

* Mentioned after individual mean if not the same as given in this column.

** The only significant difference compared with the reserpine group lies in the 5HIAA value ($p < 0.05$).

above the ligature was filled with watery content after the four hour experiment, especially in the groups given reserpine. When neostigmine alone was used, there were spasms in the intestine and often only little and relatively dry content. Thus neostigmine probably increased the tonus; in the dose used, it did not increase the movement of the intestinal content to such an extent.

Great variations in the blood values do not allow statistically significant conclusions be drawn. Even reserpine was unable to lower the 5HT content within four hours but the figure in the reserpine plus neostigmine group is the lowest of all.

The urine flow in the control groups was somewhat higher (control: 7 ml/hr, no intestinal occlusion: 6.4 ml/hr) than in the experimental groups (reserpine: 3 ml/hr, both neostigmine and neostigmine + reserpine: 4.7 ml/hr) but the differences were not significant.

There was no relationship between the 4 hr urinary 5HT and that in the blood at the time of killing. Reserpine increased 5HT excretion significantly and in the group receiving neostigmine alone a number of low values were found (mean $p < 0.05$). Neostigmine, however, was not able to prevent the increase produced by reserpine and, in fact, the result after reserpine plus neostigmine was higher than after reserpine alone.

Reserpine produced a clear increase in the excretion of 5HIAA. Neostigmine, on the other hand, did not change the excretion of this acid and when given with reserpine the amount found in the urine was less than might have been expected.

The intestinal occlusion itself seemed to produce no clear changes in the results, although there was an indication of an increase of 5HT in the intestine and 5HIAA in the urine and a decrease of 5HT in the blood.

The toxicity of reserpine and neostigmine when given together in the doses used has to be considered rather high. 1.5 mg/kg of reserpine is certainly not toxic and 1 mg/kg of neostigmine (in three doses) was not fatal to a rabbit. A combination of 1.5 mg/kg of reserpine and 0.3 mg/kg of neostigmine, however, killed the rabbits within 3 to 4 hours.

DISCUSSION

5HIAA is not as important metabolite in the urine of addult rabbits as it is in most omnivorous and carnivorous animals (8, 15). However, in rabbits an increase in the 5HIAA excreted has also been reported after reserpine (23) and tetrabenazine (17). The major part of this acid is probably derived from the 5HT in the intestine (2). In spite of probably being able to increase the pressure on the intestinal mucosa, neostigmine did not release 5HT — or at least not in sufficient quantities — to be measured as an increase in 5HIAA excretion. Even under the special conditions of reserpine action neostigmine did not increase the excretion of this acid, and there was a significant unexplainable decrease.

Several workers have come to the conclusion that 5HT acts on nervous ganglia or postganglionic cholinergic nerve fibres in the guinea-pig ileum (10, 12, 20). The stimulation of the guinea-pig intestine by 5HT is partly blocked by atropine and it has been suggested that this may be due to the antagonism to the acetylcholine liberated by nerves stimulated by 5HT (9, 19). Consequently the action of reserpine, while stimulating the gut by releasing 5HT, should be enhanced by neostigmine. On the other hand no evidence has been found in the guinea-pig ileum for a cholinergic mechanism in the 5HT release (3). A small release in the intestinal 5HT could be compensated by increased synthesis but the failure to find any increase in the excretion of 5HT and 5HIAA contradicts the assumption of an increased release of the amine by neostigmine. We had the impression that in spite of increased tonus the motility of the intestinal content was not increased or was even decreased when neostigmine alone was injected. The discussion is complicated by the fact that an active intestine, at least in guinea-pigs, is rather inhibited than stimulated by 5HT (3, 4). The closing of the small intestine alone may produce some elevation in the 5HIAA excretion in the urine.

Reserpine releases 5HT from blood platelets both *in vitro* and *in vivo* (6, 22). The failure to find any clear decrease after reserpine may be, partly, due to the release of 5HT from the stores in the alimentary tract. The lowest value was found after reserpine plus neostigmine, but even this was not significantly lower than the control mean.

Some 5HT is found in the urine (5, 11, 21). The source of this amine, however, is not known; it may be synthesised in the kidney. The increased excretion by reserpine is probably a sign that a small portion of the 5HT liberated is excreted as such. The low neostigmine results may perhaps be considered less accurate than others because in some samples activity inhibiting rat stomach strip was encountered.

The results of these experiments do not support any part for 5HT in the mechanism of action of neostigmine. The work is in line with the unpublished observations from this laboratory that cholinergic drugs and cathartics do not increase the excretion of 5HIAA in humans.

SUMMARY

The effect of reserpine (1.5 mg/kg i.v.) and neostigmine (0.2 mg/kg s.c. in divided doses) alone and in combination on the 5-hydroxytryptamine (5HT) content of the small intestine, blood and urine and on the 5-hydroxyindoleacetic acid (5HIAA) in the urine within 4 hours was studied on rabbits. An experimental ileus was produced by closing the intestine above the ileocecal junction and at the same time the urethra was ligated so as to retain the urine in the bladder. 5HT was estimated by using rat stomach preparation and 5HIAA spectrophotometrically.

The range of the intestine and blood 5HT values was large and no significant differences were found. Reserpine increased 5HT and 5HIAA excretion and neostigmine decreased the amount of 5HT in the urine. Neostigmine did not significantly increase the influence of reserpine in the reactions studied, and it significantly decreased the reserpine induced elevation of 5HIAA excretion.

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THE EFFECT OF METHIMAZOLE AND POTASSIUM
THIOCYANATE ON THE 5-HYDROXYTRYPTAMINE
CONTENT OF THE RAT THYROID GLAND

by

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The thyroid gland of some mammals, *i.e.* rats and sheep, contains 5-hydroxytryptamine (5HT) in relatively large quantities (2). If the amine concentrated in this gland serves any purpose it might be expected to be connected with the specific thyroid function. To elucidate this question we treated rats with goitrogenic substances before analysing the 5HT in the thyroids. Two agents with different mechanisms of action were used: 1) methimazole, which acts like thiouracil in preventing the iodination of tyrosine (4), and does not interfere directly with ability of thyroid tissue to concentrate iodine, and 2) thiocyanate, which reduces thyroid hormone synthesis by inhibiting the uptake of iodine by thyroid tissue (5).

METHODS

Male Sprague-Dawley rats were used for these experiments, which were carried out during November-December at room temperatures of 19–22°C. Beginning two weeks before and continuing throughout the whole experiment the rats were given the following iodine poor (about 0.04 μ g iodine/g) grain mixture: 5 parts oat, 5 parts wheat and 3 parts barley.

Short-term experiments were carried out on rats weighing about 157 g (range 150–170 g). The animals were killed within

three hours, beginning at 10 *a.m.* by cutting the throat with scissors. About 24 hours before being killed one group received 2 mg (5 ml)/animal (about 15 mg/kg) of methimazole (Lääke Oy, Turku). Another group was given potassium thiocyanate (Merck A. G., Darmstadt) 10 mg (5 ml)/animal (about 65 mg/kg) 20 hours and again 3 hours before being killed. All administrations were given by stomach tube. The solutions were made in 0.45% sodium chloride and this solution was given to the controls.

In the chronic experiments the animals were given to drink 0.45% (1) sodium chloride solution, which contained 100 mg/l methimazole, or 250 mg/l potassium thiocyanate in the experimental groups. In these groups the mean body weight at the beginning was 112 g (106–117 g) and after the 4 weeks test period about 130 g (120–155 g).

For 5HT extraction the thyroids were ground with 90% acetone and left to stand overnight at -18°C . (2). Before the evaporation of the acetone in vacuo one ml 0.001 N—HCl was added and the residue was washed once with petroleum ether. The samples were assayed biologically by using the rat stomach method (6). D-lysergic acid diethylamide (LSD) and brom—LSD (BOL) (generously supplied by Sandoz Ltd., Basle) treatment prevented the response of the stomach strip to thyroid extracts to the same degree as that of the standard 5HT. 5HT was used as creatinine sulfate (generously supplied by May & Baker Ltd., Dagenham); the doses refer to the base.

RESULTS AND DISCUSSION

The results are summarised in Table 1. In the short-term experiments no significant ($p > 0.05$) changes were produced although the mean 5HT values in the thiocyanate group were somewhat higher than in the controls. If the higher amine content in some thiocyanate animals was due to the treatment it is worth while noting that even a single large dose of thiocyanate is sufficient to reduce the iodine-uptake of the thyroid gland, as was also the case with the single methimazol dose used in this experiment (1).

The results of the chronic experiments indicate that the 5HT content of the total gland increases during methimazole treatment and probably during thiocyanate treatment too. The increase is

TABLE 1

EFFECT OF METHIMAZOLE AND POTASSIUM THIOCYANATE ON THE WEIGHT AND 5-HYDROXYTRYPTAMINE CONTENT OF RAT THYROID GLAND. EACH FIGURE REPRESENTS THE MEAN \pm S.D. OF A GROUP OF EIGHT RATS. P (CALCULATED ACCORDING TO STUDENT'S T-TEST) IS GIVEN ONLY WHEN THE RESULT IN THE TREATED GROUP IS SIGNIFICANTLY ($p < 0.05$) DIFFERENT FROM THE CORRESPONDING CONTROL VALUE. FOR FURTHER DETAILS SEE METHODS

	Controls	Methimazole	Thiocyanate
<i>Short-term Experiment:</i>			
Weight mg	12.04 \pm 0.94	11.62 \pm 1.42	12.32 \pm 0.69
5HT ng* whole Gland	51.9 \pm 12.1	53.6 \pm 14.7	63.4 \pm 18.7
5HT ng/g	4320 \pm 979	4620 \pm 1130	5160 \pm 1550
<i>Chronic Experiment:</i>			
Weight mg	17.39 \pm 2.67	42.84 \pm 11.93	24.80 \pm 5.52
		$p < 0.001$	$p < 0.01$
5HT ng/whole Gland.....	70.0 \pm 10.1	122 \pm 25.7	82.0 \pm 24.1
		$p < 0.001$	
5HT ng/g	4110 \pm 851	3100 \pm 1290	3350 \pm 824

* ng = nanogram = 10^{-9} g = 10^{-3} μ g.

not associated with the iodine uptake of the gland. In a further experiment (1) it has been shown that treating for four weeks with an equal dose of methimazole increases the iodine uptake of the thyroid gland up to fivefold, and, per weight unit of the gland, up to two-fold. The increase in the 5HT content of the thyroid is somewhat less than might be expected if it followed the weight increase of the organ. The increased vascularity and blood content of the thyroid in the treated animals cannot alone be responsible for the 5HT increase of the whole gland.

It is not known where 5HT is located in the thyroid gland. If specific cells, like enterochromaffin or mast cells are the site of this amine, then some increase in the number and/or an enlargement of these cells might be expected, following the pattern of the thyroxine secreting acinar cells.

The rat thyroid gland is able to synthesise 5HT from 5-hydroxytryptophan *in vitro* and the amount decarboxylated is increased when the monoamine oxidase inhibitor, iproniazid, is present (3). Iproniazid treatment *in vivo*, however, does not increase the amount of 5HT in this tissue, which means that the stores are fully saturated by this amine or that it is ordinarily well protected. We do not know

wether the goitrogenic substances can change the 5-hydroxytryptophan decarboxylase activity in the thyroid gland but in view of the relatively small changes in the 5HT amounts, it does not seem likely.

This experiment has given no indication that the relatively high 5HT content in the rat thyroid gland plays any part in the specific thyroid hormone production.

SUMMARY

No significant changes in the 5-hydroxytryptamine (5HT) content of rat thyroid gland were produced in a 24 hour experiment by methimazole (about 15 mg/kg per os) and potassium thiocyanate (about 65 mg/kg twice per os). When these substances were given to rats in the drinking fluid (methimazole 100 mg/l, potassium thiocyanate 250 mg/l) over a period of four weeks there was an increase in the weight of thyroid gland in both groups, the total content in 5HT being increased significantly in the methimazole group. The concentration of 5HT/g seemed to be reduced in the experimental groups but the differences were not statistically significant.

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FACTORS INFLUENCING THE RELEASE OF 5-HYDROXY-TRYPTAMINE FROM THE RABBIT INTESTINE INTO THE VENOUS BLOOD

by

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The digestive tract and its enterochromaffin cells are generally regarded as one of the most important sites of the formation and storage of 5-hydroxytryptamine (5HT) in the mammalian organism (6). The major part of 5HT in the blood is localized in the platelets, minimal amounts being found in platelet-free plasma (8, 14, 16). Obviously also this amine is derived from the digestive tract (2, 7, 13). There is, however, little information on the factors regulating the liberation of 5HT. It has been proved that elevation in intraluminal pressure increases the excretion of 5HT into the lumen of the intestine in guinea-pigs (3, 4). The experiments reported here were done in order to find out whether during an increase in the intraluminal pressure and/or peristaltic movements the liberation of 5HT also into the circulation is changed.

MATERIAL AND METHODS

25 rabbits of both sexes were used for the investigation. The animals were anaesthetised with urethane (1.5—2.0 g/kg i.v.) and artificially respirated. The abdominal cavity was opened and the spleen removed. The ileum was lifted out of the abdominal cavity and a polyethylene cannula, tip towards the intestine, was inserted into the mesenteric vein of the part of the ileum about 10 cm orally from the ileocecal junction. Before insertion of the cannula, the test animal was injected intravenously with

2000 I.U. of heparin. After cannulation the part of ileum drained by the vein was closed at both ends. Samples (3 to 4 ml) of blood were collected in silicone-coated centrifuge tubes containing 1/10 volume of 1% disodium ethylene diamine tetra-acetate. The blood for each sample was collected in about three (from 2 to 13) minutes. Between the collections the cannula was closed for 2 to 3 periods not longer than 1—2 minutes and before starting collection of the next sample the blood of the first 0.5 minute was disregarded. Attempts to get platelet rich plasma with comparable platelet counts were unsuccessful and therefore whole blood was used instead of platelets. The samples were kept at around 0°C during the whole pre-extraction period. One ml of blood was used for 5HT estimation from whole blood and the rest of the sample was centrifuged to obtain platelet-free plasma. The samples were extracted with 19 volumes of acetone (1) and the 5HT determinations were made using the rat stomach preparation (15).

At the beginning of the experiment, 2—3 samples of blood were taken from each animal for the determination of control values. The intraluminal pressure in the ligated part of the intestine was then elevated by increasing peristalsis by intravenous neostigmine methylsulfate (A. B. Leo, Hälsingborg) injection (0.4 mg/animal), or by injecting air or liquid paraffin at body temperature into the intestinal lumen. These procedures are referred to in the text as «elevation of pressure». 2—3 samples were then taken at regular intervals starting one minute after each «elevation of pressure».

During the test, the temperature of the exposed part of the intestine was kept close to the normal level by means of radiation lamps and the intestine and the mesenterium were sprinkled regularly with physiological saline at body temperature.

Five animals were given 0.05 mg/kg of reserpine (Lääke Oy, Turku) intravenously 3 hours before the test. One animal received 0.5 mg/kg 3 hours and another 24 hours before the operation. Platelet counts made occasionally did not show any clear changes during the experiments.

RESULTS

The 5HT concentration in the blood was generally higher in the first sample than in the next two samples which were usually fairly similar. The range of 5HT control values for unreserpinised animals were, excluding the first ones, in plasma from 10—70 ng/ml and in whole blood from 700—5000 ng/ml. In the majority of cases «elevation of pressure» caused an increase in the 5HT concentration in the plasma of the intestinal venous blood (Fig. 1); a 2 to 4-fold increase was observed in the first plasma sample taken within 3 to 6 min. of the injection. The established elevation of 5HT level usually persisted for 10 min. or longer. Later on during

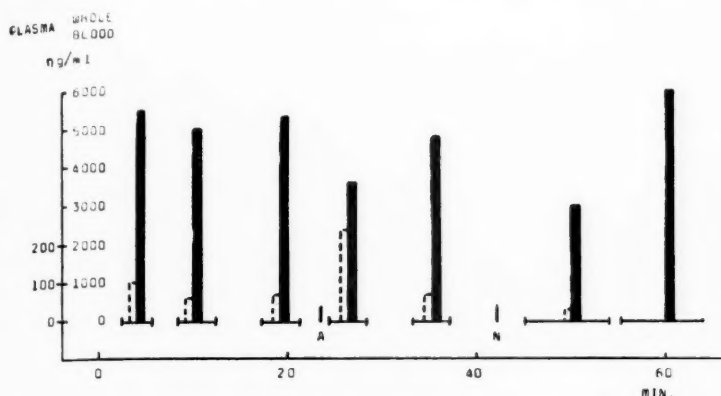


Fig. 1. — Rabbit 2.0 kg. 5-hydroxytryptamine content of plasma and whole blood of the intestinal vein in ng/ml. At 0 time the ligation of the intestinal loop.

The last plasma value not known.

Open columns — Plasma values.

Black columns — Whole blood values.

Horizontal lines — Time used for collecting the sample.

N — Neostigmine methylsulphate 0.4 mg intravenously.

A — Air into the lumen of the intestine.

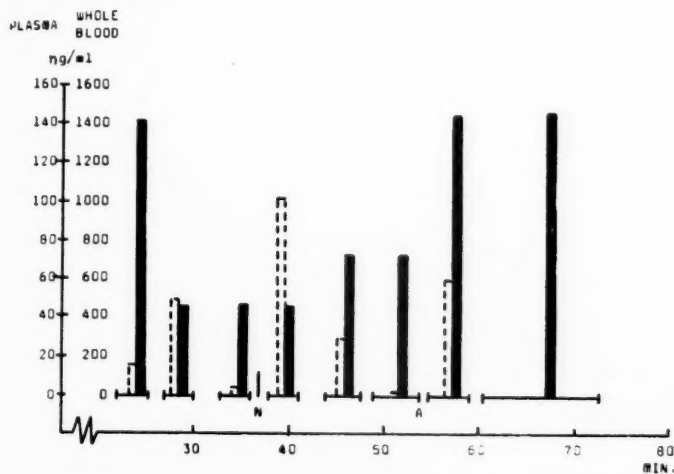


Fig. 2. — Rabbit 3.0 kg. 5-hydroxytryptamine content of plasma and whole blood of the intestinal vein. Reserpine 0.05 mg/kg intravenously 4 hours before starting the experiment. The last plasma value not known.

Open columns — Plasma values.

Black columns — Whole blood values.

Horizontal lines — Time used for collecting the sample.

N — Neostigmine methylsulphate 0.4 mg intravenously.

A — Air into the lumen of the intestine.

the experiment there was usually also an indication of 5HT rise in whole blood.

In the reserpine (0.05 mg/kg) treated animals the plasma 5HT concentrations were similar to the controls but the whole blood values were in the range of 200—1500 ng/ml. In these samples there were some high plasma values (*e.g.* the second sample in Fig. 2), which probably was due to the reserpine induced lability of the 5HT binding mechanism. In these animals «elevation of pressure» resulted in the initial rise in plasma similar to that observed in animals not treated with reserpine and the response was generally of longer duration. The large reserpine dose (0.5 mg/kg) lowered the plasma and whole blood 5HT concentration to below measurable levels (less than 1 ng/ml) in all samples.

An increase in 5HT, similar to the first increase, was observed in some cases after the second «elevation of pressure». The animals treated with reserpine showed this second increase more distinctly than the animals not given reserpine (Fig. 2).

DISCUSSION

The higher 5HT content of the first control sample in comparison with the other samples was probably due to the handling of the spleen and the intestine. The elevation of intraluminal pressure and/or peristalsis provoked either by injecting neostigmine intravenously or air or paraffin directly into the intestinal lumen, seemed to cause in the majority of cases a substantial increase of the 5HT in the plasma, and later on also in the whole blood of the vein draining that section of the intestine. There were, however, variations in the test results, which may, especially in the case of whole blood, be due to the incomplete extraction of 5HT by acetone (9, 13).

Even a small increase in plasma 5HT concentration is more striking than in whole blood where the change can only be demonstrated after greater release. That the increase also occurred following splenectomy indicates that the free 5HT most probably comes from the intestine.

In the reserpinised animals the 5HT release into plasma after «elevation of pressure» was more marked than in animals without reserpine. Reserpine inhibits the binding of 5HT with platelets and

other tissues (5, 11) and it is possible that the plasma values in reserpinised animals were higher because the platelets were not any more able to remove the amine from the plasma.

In unanaesthetised rabbits neostigmine failed to increase the excretion of 5-hydroxyindoleacetic acid (5HIAA) (10), which speaks against any greater release of 5HT after this agent in these animals. It must be born in mind, however, that 5HIAA is not the main metabolite of 5HT in rabbit (6). In the connection of the present work it is relevant to mention that in persons with various diseases of the alimentary tract, *e.g.* biliary disorders and spastic colon, considerable short term elevations in the excretion of 5HIAA has been observed (12).

SUMMARY

When the intraluminal pressure and/or motility in a part of ileum of anaesthetised splenectomised rabbits was elevated, the plasma 5-hydroxytryptamine content of venous blood from this portion was transiently increased 2 to 4-fold.

Pretreatment with small doses of reserpine seemed to augment this reaction.

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NEW ASPECTS IN THE 5-HYDROXYINDOLEACETIC
ACID EXCRETION AFTER ADMINISTRATION OF
5-HYDROXYTRYPTAMINE

by

M. M. AIRAKSINEN, M. K. PAASONEN and P. PELTOLA

(Received for publication February 16, 1960)

5-Hydroxyindoleacetic acid (5HIAA) is considered to be the main metabolite of endo- and exogenous 5-hydroxytryptamine (5HT) in most mammals (3, 4, 14). 5HT itself is excreted in small quantities in urine (1, 7, 13). In previous experiments on human subjects, however, no significant increase in the excretion of 5HIAA was found following a single intravenous injection of 4.3 mg of 5HT (12). In the majority of experiments with human subjects, when 5HIAA excretion has been noticed, it has been given slowly intravenously or subcutaneously. The present investigation was made to find out whether the route of administration of 5HT has any influence on the excretion of 5HT itself and 5HIAA in rats.

MATERIAL AND METHODS

Male Sprague-Dawley rats of the average weight of 170 g were used. They were kept on standard food without any fast until the experiment, which was done at room temperature of about 22°C. The animals were placed separately in metabolism cages and were then given twice within an hour by stomach tube tap water a volume equal to about 3.5% of the body weight and then a control urine sample of 4 hours was collected. After that 5HT was injected subcutaneously or intravenously (in 2 ml/kg) and the same amount of water was given again. The last water administration was made 4 hours later and the urine collected from a period of 8 hours following the 5HT injection. The rats in the non-pretreated group receiving

1 mg/kg of 5HT had the water loading at two hour intervals. When reserpine (generously supplied by Lääke Oy, Turku) was used a dose of 5 mg/kg was injected intraperitoneally 48 hours before the experiment.

5HT was assayed from the urine by using rat stomach method (16). The urine of the hydrated rats has been found suitable for 5HT estimation without any treatment (Airaksinen and Uuspää, to be published). 5HIAA was measured spectrophotometrically (15). 5HT (generously supplied by F. Hoffmann-La Roche & Co, Basle) was used as creatinine sulphate; the doses refer to the base.

RESULTS

The main results are given in Table 1. The basic excretion of 5HT under these conditions has been about 0.08 μ g/hr and that of 5HIAA about 6 μ g/hr. The excretion rates of 5HT and 5HIAA during the 12 hr experiment without treatment have been fairly constant.

TABLE 1

EFFECT OF THE ROUTE OF ADMINISTRATION OF 5-HYDROXYTRYPTAMINE (5HT) ON THE AMOUNT OF 5HT AND 5-HYDROXYINDOLEACETIC ACID(5HIAA) IN THE RAT URINE DURING 8 HOURS. MEANS \pm STANDARD DEVIATIONS. P VALUES ARE GIVEN ONLY WHEN THE RESULTS AFTER INTRAVENOUS AND SUBCUTANEOUS ADMINISTRATION ARE SIGNIFICANTLY ($P < 0.05$) DIFFERENT ACCORDING TO STUDENT'S T-TEST. FOR FURTHER DETAILS SEE TEXT

Number of Animals	Pre-treatment	5HT	Per Cent of Injected 5HT Found in Urine as	
			5HT	5HIAA
4	None	1 mg/kg I.V.	0.63 \pm 0.10	35.1 \pm 5.6
4		" S.C.	1.76 \pm 0.87	67.0 \pm 8.9
			$p < 0.05$	$p < 0.01$
4	"	10 mg/kg I.V.	1.46 \pm 0.87	23.6 \pm 9.9
4		" S.C.	0.76 \pm 0.84	47.2 \pm 9.9
				$p < 0.01$
5	Reserpine	1 mg/kg I.V.	0.72 \pm 0.48	49.3 \pm 19.8
6	"	" S.C.	0.69 \pm 0.47	52.3 \pm 24.8

About twice as much 5HIAA was excreted after subcutaneous than after intravenous injection in both *unreserpinised* groups. The excretion of 5HT also was significantly higher after subcutaneous than after intravenous injection when a dose of 1 mg/kg was used. The urinary excretion was almost blocked for the first 4 hours after subcutaneous doses but much less after the intra-

venous injections. The total amount of urine excreted within 8 hours, however, was about the same in both groups. After the 1 mg/kg dose urine was analysed in two hour intervals (not indicated in the table) and the excretion of both 5HT and 5HIAA had reached usually the preinjection level in about 6 hours.

When 1 mg/kg of 5HT was injected after *reserpine* treatment the amount excreted as 5HIAA was almost the same regardless of the method of administration. Also the urine flow was quite similar in both groups.

DISCUSSION

After subcutaneous administration of 5HT the level of this amine has been found to remain elevated in rat serum up to 48 hours (4). On the other hand, after intravenous injection of 5HT into rats less than 10% could be found after 5 minutes in the whole blood (10). It is possible that after an intravenous dose much of the amine is taken by other tissues because the platelets have been unable to bind so much in a short time. This could influence the metabolism of the amine leading to formation of derivatives other than 5HIAA.

The absorption of 5HT from the subcutaneous injection site is slow (4) and hence the platelets will be able to take the amine entering the blood and will then protect it from the «fate» of the intravenously administered 5HT. It is also known that platelet-rich rabbit plasma is able to destroy 5HT released from the platelets *in vitro* by *reserpine* (11). It might be possible that mechanisms which do not normally play any important part in the 5HT metabolism will enter the picture when the concentration of this amine in plasma is unusually high as after intravenous injection.

After *reserpine* platelets lose their 5HT and they become unable to take it from the surrounding fluid (2, 8, 9, 11). The similarity in the 5HIAA excretion patterns in the *reserpinised* animals of both groups points to the importance of the functioning platelets in the phenomenon under discussion. We do not know, however, the role of the other tissues, whose 5HT binding capacity is also impaired by *reserpine*.

If the probably lower 5HT content of blood during the first four hours after an intravenous injection would not explain the

smaller anti-diuretic effect, one could assume that the tryptamine receptors involved will become saturated by the initial high 5HT concentration and insensitive for the further 5HT action, like in the intestinal plain muscle (6).

The results reported are in full accordance with those of Erspamer (3,5), who has shown that after intravenous injection 5HT is several times less active as an anti-diuretic than after subcutaneous injection. Whether the explanation for the phenomenon reported lies in the platelets remains to be seen. Further work on the problem is in progress.

SUMMARY

5-Hydroxytryptamine (5HT) was injected subcutaneously and intravenously (1 mg/kg and 10 mg/kg) in water-loaded rats and the excretion of the injected amine was estimated in the urine as 5HT and 5-hydroxyindolacetic acid (5HIAA) during 8 hours. The amount excreted as 5HT was negligible as compared to that of 5HIAA. After subcutaneous injection twice as much of 5HIAA was excreted than after a similar dose given intravenously. After the smaller dose there was a similar ratio in the 5HT excretion as well in the two groups. When the rats were treated with reserpine (5 mg/kg intraperitoneally) 48 hours before injecting 1 mg/kg of 5HT, no such difference could be demonstrated.

Acknowledgements. — This work was supported by a grant from the State Commission for Natural Sciences.

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AGGLUTINATION OF M. RHESUS ERYTHROCYTES BY HUMAN HEPATITIS SERUM

by

S. MATTILA

(Received for publication December 28, 1959)

The observation that human hepatitis sera agglutinated M. rhesus cells in higher dilutions than control sera was first described by Hoyt and Morrison (2). The purpose of this work has been to further investigate this phenomenon. The sera of a group suffering from different diseases, leptospirosis mononucleosis, in addition to the sera of healthy blood donors were tested for comparison.

MATERIALS

The hepatitis sera were taken from patients of the urban Hospital of Communicable Diseases in Helsinki. The diagnosis of all the cases had strong clinical and laboratory support. The blood Service Center of the Finnish Red Cross donated the sera of healthy blood donors. The mononucleosis and leptospirosis sera were taken from materials of our Department. In the two former groups the sera were tested not later than two days after obtaining the sera. Most of the mononucleosis sera had been stored some weeks and some of the leptospirosis sera had been more than two years in storage (in ice).

METHOD

M. rhesus red blood cells were collected in A.C.D. solution and stored in a refrigerator. A working suspension was prepared by

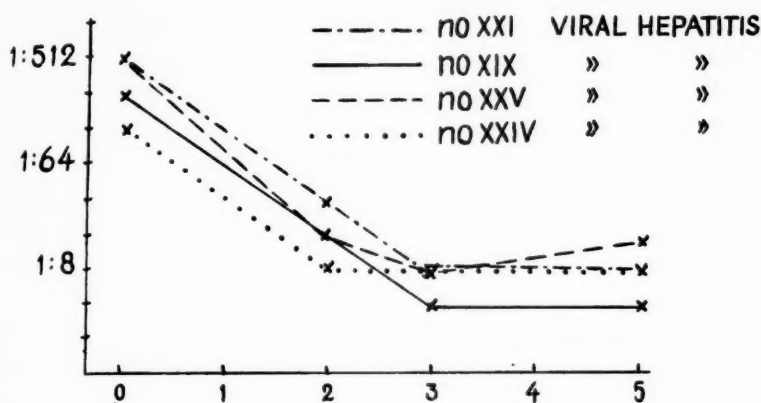
Sample n:o	Haemagglutination + (the highest dilution)				
	Viral hepatitis	Blood donors	Different diseases	Lepto- spirosis	Mono- nucleosis
I	1: 64	1: 32	1: 128	1: 64	1: 32
II	1: 32	1: 16	1: 16	0	1: 32
III	1: 128	1: 32	1: 32	0	1: 32
IV	1: 16	1: 16	1: 16	1: 2	1: 64
V	1: 256	1: 8	1: 8	1: 2	1: 256
VI	1: 512	1: 8	1: 8	1: 16	1: 16
VII	1: 256	1: 32	1: 2	1: 16	1: 64
VIII	1: 16	1: 8	1: 4	1: 32	1: 256
IX	1: 128	1: 8	1: 2	1: 2	
X	1: 32	1: 32	1: 2	1: 16	
XI	1: 64	1: 4	0	1: 8	
XII	1: 32	1: 16	1: 64	0	
XIII	1: 16	1: 4	1: 32	1: 32	
XIV	1: 32	1: 32	1: 32	1: 8	
XV	1: 16	1: 16	1: 32	0	
XVI	1: 512	1: 64	1: 16	1: 32	
XVII	1: 128	1: 512	1: 16	0	
XVIII	1: 16	1: 8	1: 64		
XIX	1: 256	1: 128	1: 16		
XX	1: 128	1: 4	1: 16		
XXI	1: 512	1: 64	1: 64		
XXII	1: 128	1: 8	1: 8		
XXIII	1: 256	1: 8	1: 4		
XXIV	1: 128	1: 16	1: 2		
XXV	1: 512	1: 32	1: 8		
XXVI		1: 16	1: 16		
XXVII		1: 32	1: 64		
XXVIII		1: 4	1: 64		
XXIX			1: 4		
XXX			1: 16		
XXXI			1: 16		
XXXII			1: 512		
XXXIII			1: 16		
XXXIV			1: 4		
XXXV			1: 32		
XXXVI			1: 256		
XXXVII			1: 32		
Mean titer	1: 167	1: 41	1: 44	1: 14	1: 94

washing the cells three times in saline, resuspending them after the last washing to make up a 1 per cent suspension by volume. The working suspension was made just before using, and unused portions were discarded. The cells were not used after being seven days in storage. The sera were diluted in powers of two by saline. The same amounts, 0.2 ml, of these dilutions and of the cell suspension were mixed in test tubes. The agglutinations were estimated after two hours storage in a refrigerator. In uncertain cases a microscopic examination was performed.

RESULTS

The agglutination titers in the different groups are collected in the following table:

The effect of the storage of the sera on the agglutination was investigated with some hepatitis sera. These were tested for agglutination at short intervals. Fresh rhesus cells were used every time to eliminate effects due to gradual loss of their agglutination capacity caused by storage (2). The results are described in the following figure:



These examples show that the titers fall after 2—3 weeks storage to a constant level. This seems to be the the general trend with other sera too.

DISCUSSION

It is evident that the mean titer in the hepatitis group is remarkably higher than in the other groups. The overlapping however seems to be great between the different groups. In the mononucleosis group there was a relatively high mean titer also. Their titers would probably have been still higher if the sera had been tested fresh. The somewhat low titers in the leptospirosis group are probably due to the storage of the sera also.

The mechanism of the agglutination is probably a nonspecific reaction. The falling of the titers in storage indicates an enzyme-like structure. The possibility of a direct virus agglutination seems us small because there might be too few viruses in the dilutions to cause agglutination.

Compared with the results of other investigators the results of the author agree with those of Colobert *et al.* (1). Because of the overlapping, the test can hardly be used in clinical practice.

SUMMARY

Haemagglutination of M. rhesus cells with human viral hepatitis sera was investigated. The sera of healthy blood donors as well as the sera of some patients suffering from leptospirosis, mononucleosis and different diseases were tested for comparison. A higher mean titer in the hepatitis group and a relatively high mean titer in the mononucleosis group was observed. Some high titers in the other control groups were also found. Because of this overlapping the reaction cannot be regarded as specific for viral hepatitis. The falling of the titers of sera in storage indicates that the haemagglutination could be due to enzyme-like reagents.

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THE HISTOCHEMICAL DEMONSTRATION OF CARBONIC ANHYDRASE

AN ATTEMPT TO LOCALIZE ITS INHIBITION BY ACETAZOLAMIDE
(DIAMOX^(R)) IN RAT KIDNEY

by

KIMMO K. MUSTAKALLIO, JYRKI RAEKALLIO, and
EEVA RAEKALLIO,

(Received for publication January 14, 1960)

The histochemical method of Kurata for the demonstration of carbonic anhydrase (5) has been reported to be unreproducible by Wachstein (10), Häusler (4), Fand *et al.* (2), Telkkä and Kuusisto (9), and by us (7). Even Braun-Falco and Rathjens (1), Goebel and Puchtler (3), and Puchtler and Ranninger (8) complained about the haphazard unspecific precipitation of cobaltous sulfide on the sections. In addition, they were unable to demonstrate inhibition of carbonic anhydrase by acetazolamide (Diamox^(R)), a potent inhibitor of this enzyme (6). In a reappraisal of Kurata's method, Fand *et al.* (2) claimed that the finally demonstrated cobaltous sulfide is not due to enzymatic enhancement of the formation of cobaltous carbonate by carbonic anhydrase, but rather to the presence of locally high tissue concentrations of divalent cations.

Recently Häusler (4) published a new modification of the method for histochemical demonstration of carbonic anhydrase, which he claimed to be specific, since the reaction was reported to be inhibited by acetazolamide *in vitro*. In our preliminary experiments applying this method in the study of carbonic anhydrase inhibition by acetazolamide in rat kidney, we found that the

final staining was superior to that achieved by the method of Kurata (5). These experiments, however, revealed serious pitfalls in this method, which enforced us to check its reliability.

METHODS AND RESULTS

The histochemical technique for carbonic anhydrase as described by Häusler (4) was scrupulously followed. Fig. 1 depicts the result in a kidney of an untreated control rat after incubation for 90 minutes. Acetazolamide produced a slight to moderate reduction in the staining, but first after a huge subcutaneous dose of 1 000 mg per kg of body weight (Fig. 2). The rats were killed by decapitation five hours after the administration of the drug. In the corresponding dosage, chlorothiazide and dihydrochlorothiazide had no effect on the staining. The diuretics were dissolved in a solution containing 80 per cent N, N'-dimethylacetamide and 20 per cent distilled water. This solvent did not change the staining in any way.

The unexpectedly slight inhibition of carbonic anhydrase by huge *in vivo* doses of acetazolamide prompted us to check the specificity of the method of Häusler.

Preincubation of sections for one hour in a 8×10^{-3} M solution of sodium acetazolamide, in a 10^{-5} M solution of potassium cyanide, or in water of 80°C resulted only in a slight diminution of staining comparable to that achieved by the administration of huge doses of acetazolamide to rats. These experiments suggest that the staining is mostly of nonenzymatic nature.

A simple cation exchange mechanism of staining in Kuratas' method was demonstrated by Fand and her coworkers (2) by the use of a Coujard model slide technique. This technique as such could not be adapted in our study, since a prerequisite for succesful staining in the method of Häusler is the floating of sections on the incubation medium. We modified the technique by dissolving zinc sulfate and the chlorides of cadmium, manganese, calcium and magnesium in 2½ per cent aqueous gelatine at a final cation concentration of one per cent. After the gelatine was congealed it was cut in small blocks, which were sectioned with a cryostat-microtome at 15 microns just as the kidneys. Manganese, calcium and magnesium caused a disintegration of gelatine, whereas zinc



Fig. 1. — Distribution of cobaltous sulfide staining in a 15μ cryostat section of rat kidney incubated for carbonic anhydrase according to Häusler (4).

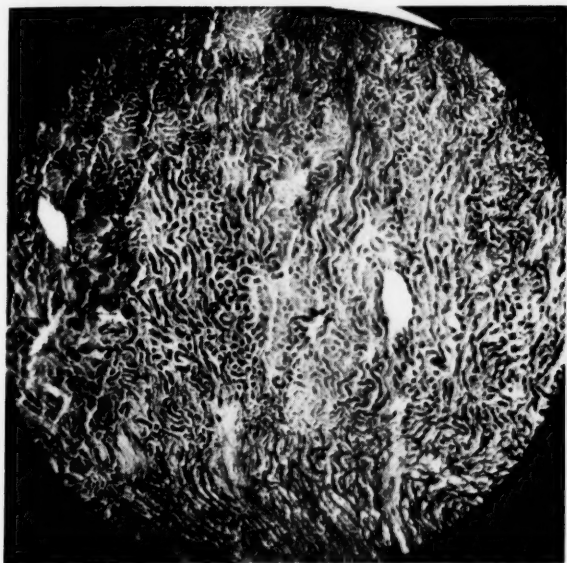


Fig. 2. — A moderate reduction in the staining intensity 5 hours after a subcutaneous 1000 mg per kg of body weight dose of acetazolamide (Diamox®). The diminution seems to be most pronounced in the outermost cortical zone of rat kidney.



Fig. 3. — Nonspecific staining of a zinc-gelatine control section incubated for carbonic anhydrase according to Häusler (4).

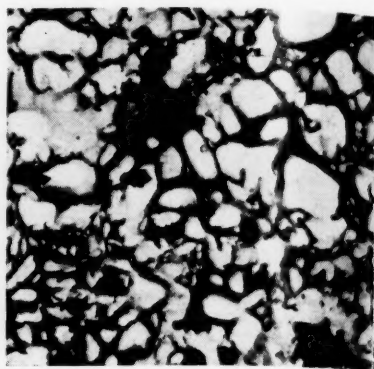


Fig. 4. — Nonspecific staining of a cadmium-gelatine control section incubated for carbonic anhydrase according to Häusler (4).

and cadmium gelatines were successfully sectioned and incubated floating on the mixture according to Häusler. The results for zinc and cadmium are seen in Figs 3 and 4, respectively. This nonspecific staining seems to be due to cation exchange, but since cobaltous carbonate forms a tiny film on the incubation mixture when incubation is prolonged, a direct attachment of cobaltous carbonate on the sections must also be taken in consideration. Subsequent washings removed not adequately this nonspecific staining. Thus the very circumstances recommended by Häusler, namely the flotation of sections on the incubation medium, seem to be an important pitfall in his method.

SUMMARY

A reappraisal of Häusler's method for the histochemical demonstration of carbonic anhydrase revealed that the staining was mostly nonenzymatic in nature. The nonspecific staining of zinc- and cadmium-gelatine model sections brought indirect evidence for a cation exchange between divalent cations pre-existing in the floating kidney sections and cobaltous carbonate concentrating on the surface of incubation medium. Huge subcutaneous doses of acetazolamide were required to suppress slightly the staining in kidney cortex of rat.

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TWO COMBINED PERTUSSIS-DIPHTHERIA-TETANUS PROPHYLACTICS

A COMPARATIVE STUDY WITH DIFFERENT CONTENTS OF DIPHTHERIA
TOXOID AND ADJUVANT

by

L. JÄNNES, TIMO KOSUNEN, and P. LEISTI

(Received for publication December 30, 1959)

The combined PDT prophylactic generally used in Finland since 1956 is prepared to contain the equivalent of 20 000 million *Bordetella pertussis* bacteria, 50 Lf doses of purified diphtheria toxoid, 10 Lf doses of purified tetanus toxoid and 10 mg of aluminium phosphate adjuvant (2.2 mg of aluminium) per ml.

To achieve a basic immunity in the population, children are vaccinated with this prophylactic at the age of 3 to 6 months by giving three intramuscular injections (0.5 ml each) at 4 to 6 weeks' intervals.

Considering the relatively large amounts of diphtheria toxoid and aluminium phosphate given in each injection, an experimental prophylactic with reduced contents of these two components was prepared.

The effect of these reductions on the diphtheria antitoxin response of children was studied in a small-scale field trial.

MATERIAL AND METHODS

Prophylactics. — 1. The generally used prophylactic contained 50 Lf doses of diphtheria toxoid, 10 Lf doses of tetanus toxoid, the equivalent of 20,000 million pertussis bacteria and 10 mg of aluminium phosphate (2.2 mg of Al) per ml.

2. In the experimental prophylactic the diphtheria toxoid content was reduced to 25 Lf doses per ml and the aluminium phosphate content to 5 mg (1.1 mg of Al) per ml. Otherwise both prophylactics were identical and all four components were taken from the same batches. Both toxoids were highly purified.

Immunisation. — 29 children aged from 3 to 12 months in an orphanage were immunised, 12 with the generally used prophylactic and 17 with the experimental prophylactic. Two injections of 0.5 ml were given intramuscularly into the gluteal region at an interval of 5 weeks.

Determination of the Antitoxin Response. — Blood samples were drawn immediately before and 6 weeks after the second immunisation. The diphtheria antitoxin level of the samples was assayed by the guinea pig test method according to Römer (2), carrying the assays on the 1/1000 IU antitoxin level.

RESULTS

The diphtheria antitoxin titres in the sera of the children five weeks after the first immunisation are shown in Fig. 1. Only a few children had detectable amounts of diphtheria antitoxin at this stage. In one case the titre was exceptionally high; this child was one of the oldest studied.

Fig. 2. shows the antitoxin titres six weeks after the second immunisation. All the children showed a definite antitoxin response, the average titre of diphtheria antitoxin being in both groups on the level of 0.1 IU/ml of serum.

In five children a slight rise in the body temperature (1° C) was observed on the day following the vaccination. Three of these children were immunised with the generally used prophylactic and two with the experimental one.

and thus a reduction of the total reactive antigenic material in the prophylactic might still be possible. If the mineral adjuvant content also could be reduced, an additional decrease especially in the number of local reactions caused by the prophylactic would be probable.

Our results seem to indicate that the response to diphtheria toxoid immunisation is not altered significantly although the concentration of diphtheria toxoid and mineral adjuvant in the combined prophylactic are reduced to one-half. Even the fact that the concentration of tetanus toxoid in the experimental prophylactic was kept unaltered and, consequently, the ratio between diphtheria and tetanus toxoids (Lf/ml) was changed from 5:1 to 2.5:1, had no effect on the diphtheria antitoxin response.

Considering that the presence of the pertussis moiety in a prophylactic is deemed to require three successive inoculations, this study seems to indicate that there is a possibility of a considerable reduction of the amount of diphtheria toxoid and mineral adjuvant in a combined vaccine of this type. Already after two inoculations with the diluted experimental vaccine the diphtheria antitoxin titre in the serum of immunised children rose well above the Schick conversion level (1).

The number of vaccinated individuals in this trial was too small to provide a possibility to see the effect of the dilution of the prophylactic on the number and nature of untoward vaccination reactions.

SUMMARY

The possibility to reduce the diphtheria toxoid and aluminium phosphate adjuvant content of a combined pertussis-diphtheria-tetanus prophylactic was studied. A 50 per cent reduction in the concentration of these two components was made without an effect on the diphtheria antitoxin response of the immunised children.

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RESPONSE OF YOUNG MICE OF IMMUNISED PARENTS TO TETANUS TOXOID

by

TIMO KOSUNEN and PEKKA HALONEN

(Received for publication June 2, 1960)

Studies on the effect of passive maternal immunity on the vaccination response in young children have mostly been based on determination of antibody level (1, 3, 5, 7, 11, 13, 14). The same is true of similar studies in experimental animals (2, 8). In the present study the vaccination response has been followed by toxin challenge, which may yield a more reliable idea of true acquired immunity. The immunising effect of tetanus toxoid has been studied by this toxin challenge method in young mice of immunised parents.

MATERIAL AND METHODS

Mice. — Animals of the Swiss albino Webster strain were used.

Tetanus toxoid was prepared by the standard method in a modified Mueller—Miller medium (9), concentrated and purified to contain 900 Lf/ml and 1000 Lf/mg protein. The final preparation contained 10 Lf of tetanus toxoid and 2 mg of aluminium phosphate/milliliter.

Tetanus toxin was prepared in the same modified Mueller—Miller medium. The dilutions of the toxin were made with 10% peptone solution.

Plan of Study. — Fig. 1 shows the schedule for the first series of experiments. Two days after mating the males and females

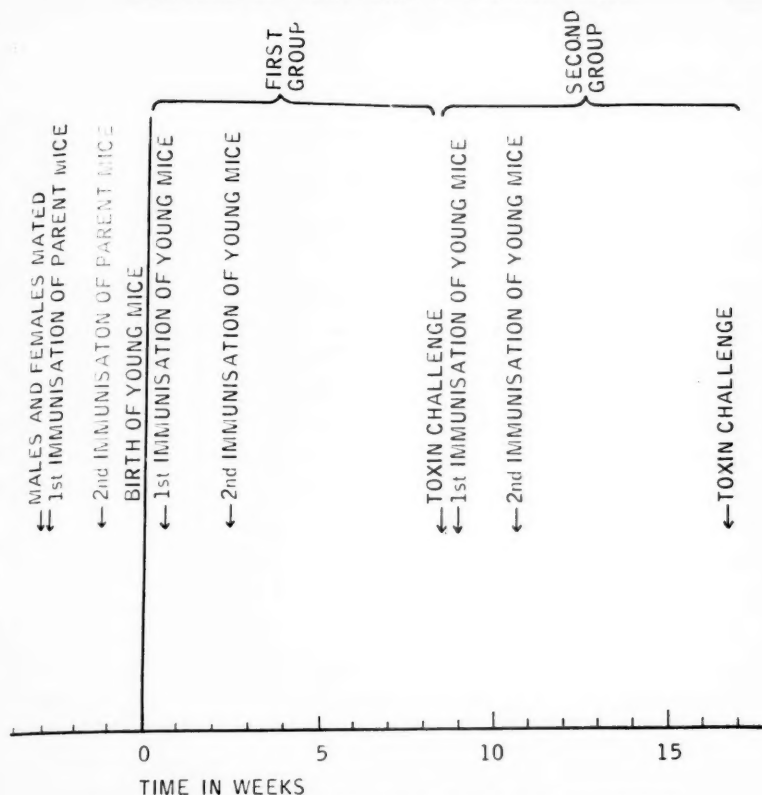


Fig. 1. — The schedule for the first series of experiments.

were divided into two groups, one of which was immunised twice with tetanus toxoid at an interval of 12 days. The immunisation was performed by subcutaneous injection of toxoid diluted with saline to contain 1 Lf in 0.5 ml.

Some of the offsprings of both the immunised and the non-immunised parents were immunised for the first time 2—5 days after birth, and a second time 12 days later. Both immunisation doses given to the young were 0.2 Lf of tetanus toxoid in 0.1 ml saline. Six weeks after the second immunisation the immunity of the young of both parental groups was determined by injecting 0.2 ml of 10-fold dilutions of tetanus toxoid subcutaneously. Another group of the offsprings of both the immunised and non-immunised parents was immunised for the first time at the age of 8 weeks. The second immunisation was performed after 12

days and the toxin challenge was given 6 weeks after the second immunisation.

In the second series of experiments the same immunised and non-immunised males and females were mated again 3 months after the first mating. The young that were born were toxin-challenged in the same way as the first group of young in the first series.

RESULTS

Immunity of the Young. — The decrease in immunity of the offspring of immunised parents is shown in Table 1. When 2–5 days old, the young tolerated an amount of toxin that was 10,000 times larger than the dose tolerated at the same age by the young of non-immunised parents. At the age of 15 weeks the toxin tolerance of the immunised and non-immunised young did not differ significantly.

The second series of experiments was made on the young born 14 weeks after the second immunisation of the parents. At the age of 2–5 days these young tolerated a dose of toxin 100 times as great as the amount tolerated by the young of non-immunised parents, but as little as 8 weeks later the toxin tolerance was practically the same in the two groups.

TABLE 1
SURVIVAL RATE OF YOUNG MICE OF IMMUNISED AND NON-IMMUNISED PARENTS
AFTER VARIOUS DOSES OF TETANUS TOXIN AT VARIOUS AGES

Amount of Toxin	Age of the Young Mice							
	2–5 Days		6 Weeks		8 Weeks		15 Weeks	
	Immu- nised Parents	Non- immu- nised Parents	Immu- nised Parents	Non- immu- nised Parents	Immu- nised Parents	Non- immu- nised Parents	Immu- nised Parents	Non- immu- nised Parents
0.1 ml	0/1							
0.01 "	1/1 ¹							
0.001 "	1/1		0/2					
0.0001 "			2/2 ¹		0/2		0/2	
0.00001 "		0/1	2/2	0/2	2/2 ²	0/2	2/2 ²	2/2
0.000001 "		0/1 ¹		1/2 ¹		2/2 ¹	2/2 ²	2/2
0.0000001 "		1/1		2/2		2/2		2/2

¹ one paralysed

² two paralysed

TABLE 2

SURVIVAL RATE OF IMMUNISED YOUNG MICE OF IMMUNISED AND NON-IMMUNISED PARENTS AFTER VARIOUS DOSES OF TETANUS TOXIN. THE FIRST GROUP WAS IMMUNISED AT THE AGE OF 0 AND 2 WEEKS, THE SECOND AT THE AGE OF 8 AND 10 WEEKS. TOXIN CHALLENGE WAS GIVEN SIX WEEKS AFTER THE IMMUNISATION

Amount of Toxin	First Group of Immunised Young Mice			Second Group of Immunised Young Mice		
	Immunised Parents	Non-immunised Parents	Controls	Immunised Parents	Non-immunised Parents	Controls
0.1 ml		0/2		0/2	2/2 ³	
0.01 "		0/2		1/2 ¹		
0.001 "		2/2		2/2		
0.0001 "	0/2	2/2				
0.00001 "	2/2 ²		0/2			2/2 ²
0.000001 "			1/2 ¹			2/2
0.0000001 "			2/2			2/2

¹ one paralysed

² two paralysed

³ 1.0 ml of toxin 0/2

Response to Tetanus Immunisation. — Table 2 shows the response to immunisation in the young of both the immunised and the non-immunised mice. When immunisation was begun immediately after birth, the young of immunised parents at the age of 8 weeks tolerated an amount of toxin which was one hundredth of that tolerated by the young of non-immunised parents. On the other hand, the toxin tolerance of these immunised young mice was the same as that of the 8 week old mice shown in Table 1.

In other words, the toxin tolerance of the immunised young of immunised parents, as shown in Table 2, was not appreciably influenced by the immunisation of the latter.

When immunisation of the young was begun at the age of 8 weeks, the difference in toxin tolerance between the offspring of immunised and non-immunised parents was only tenfold. The toxin tolerance of these young of immunised parents was one thousand times greater than the tolerance of 15 week old non-immunised young of immunised parents.

It is also seen from Table 2 that the response of the young of non-immunised parents to immunisation was better in the older group of young. In the group immunised for the first time

at the age of 2—5 days there was a difference in toxin tolerance of 1:1.000 as compared with the controls, whilst the difference was 1:10.000 in the group immunised for the first time at the age of 8 weeks.

DISCUSSION

The more widely adopted the vaccination of young children becomes, the more important becomes the question of how immunity transferred from the mother influences the response to vaccination. The need for a thorough acquaintance with these immunological relationships is all the more urgent because in many countries it is regarded as desirable to vaccinate children against certain diseases, *e.g.* pertussis and polio, as early as possible (1, 4, 6, 10, 12). On the other hand, vaccination against polio during pregnancy, for instance, has also been recommended (6).

The data in the present study indicate that the response of young mice to tetanus immunisation was clearly depressed by passive maternal immunity. In the first series of experiments, where immunisation of the young was begun a few days after birth, no response to immunisation was found. When immunisation was begun 8 weeks later, a response was observed, although it was weaker than in the young of non-immunised parents.

However, in evaluating early vaccination it is interesting to note that in those young of non-immunised parents in which immunisation was begun only a few days after birth, the response was only slightly weaker than in the young immunised for the first time at the age of 8 weeks.

SUMMARY

The passive immunity of the offspring of mice immunised during pregnancy with tetanus toxoid and its effect on their response to immunisation with tetanus toxoid were studied, with the following results:

1. Immediately after birth the young tolerated an amount of toxin which was 10,000 times that tolerated by the controls. After 15 weeks the difference was no longer significant.

2. When immunisation of the young was begun at the age of 2-5 days, no response was found.
3. When immunisation of the young was begun at the age of 8 weeks, there was a response, although it was weaker than in the controls.

When immunisation of the controls was begun at the age of 2-5 days the response was slightly weaker than when immunisation was begun at the age of 8 weeks.

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EFFECT OF NEOMYCIN ON STAPHYLOCOCCAL INFECTION IN MICE

by

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Neomycin is effective against both gram-negative and gram-positive bacteria. In the present study, the effect of neomycin on experimental *Staphylococcus aureus* infection in mice was evaluated by the quantitative microbial enumeration method, first described by Smith and Dubos (3).

MATERIAL AND METHODS

Mice. — 120 male mice of the Swiss Albino Webster strain were used. Their weights ranged from 18 to 20 g at inoculation.

Staphylococcus aureus strain «Orion», phage pattern 29/73 (/7/42E/54), was used. It is coagulase (human and mouse plasma) and phosphatase producing, non-motile, produces orange-yellow pigment, forms acid from glucose, lactose and mannitol, causes no hemolysis on blood agar, grows in media containing 10 per cent of sodium chloride. The strain is sensitive to a penicillin concentration of 0.4 units/ml and to a neomycin concentration of 0.5 γ /ml.

Neomycin. — The neomycin sulphate («Fradiomycin») used was a lot kindly placed at our disposal by the manufacturer, Meiji Seika Kaisha Ltd, Tokyo. The neomycin activity of the powder was stated to be 650 γ /mg. The antibiotic was given subcutaneously in sterile saline, in individual doses of 0.5 ml.

Technique. — The mice were inoculated intravenously with 0.2 ml of a 1:2 dilution of a 24-hour broth culture of bacteria. At appropriate intervals 5 treated and 5 control animals were sacrificed, the kidneys removed and ground with sterile saline in pyrex glass tubes with Teflon homogenisers. The number of culturable staphylococci per ml of tissue was determined by making serial dilutions from homogenates on agar plates and counting the bacterial colonies grown during incubation. The enumeration method has been described in detail earlier (2).

The study consisted of two experiments. In both experiments 30 mice were treated with neomycin and 30 were controls. In the first experiment, two doses of 2 mg of neomycin sulphate/mouse were given; the first one hour after the bacterial inoculation, the second on the fifth post-challenge day. In the second experiment, 1 mg of neomycin sulphate/mouse was injected every two days. The first injection was given one hour after the inoculation of the bacteria. The control animals were given 0.5 ml of saline.

RESULTS

First Experiment. — The logarithms of the number of culturable staphylococci found in the kidneys are given in Fig. 1. Table 1 shows the daily means and the statistical data of the results. In the control group the bacteria increased until the fifth post-challenge day; after that the microbial population curve had a declining trend. The microbial population curve of the neomycin group was lower than that of the control group on the first post-challenge days. The rapid initial fall in the bacterial population after the third post-challenge day, however, was followed by a secondary rise. This was reflected also in the statistical evaluation of the results, since on the seventh and tenth post-challenge day the differences between the groups were not significant.

Second Experiment. — The quantities of bacteria found in the kidneys are shown in Fig. 2. The daily means and the statistical data of the results are given in Table 2. The slowly declining trend in the control group reveals the variation in the bacterial population. In the neomycin group the number of bacteria decreased rapidly. From the first post-challenge day on the microbial population curve of this group was lower than that of the control group, and

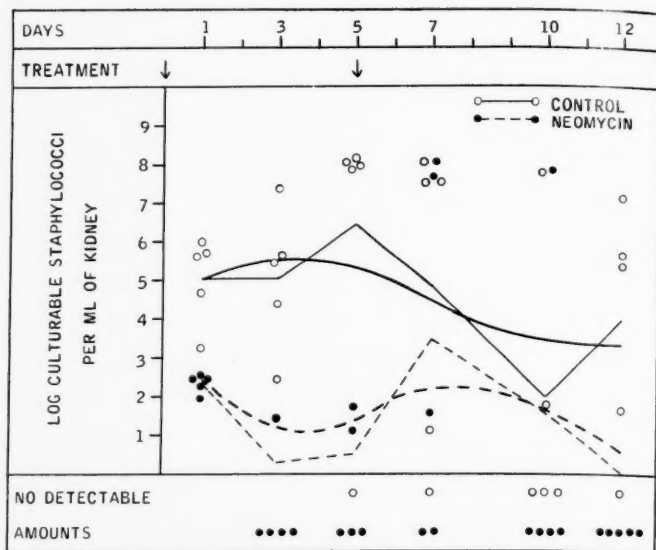


Fig. 1. — Diagram showing the logarithms of the number of culturable staphylococci per ml of kidney in control mice and in mice treated with neomycin (experiment 1). Each circle represents one control mouse, the hairline connects the daily means and the thicker line shows the trend. Each solid dot represents one treated mouse, the broken hairline connects the daily means and the thicker broken line shows the trend. See also Table 1.

TABLE 1

FIRST EXPERIMENT. LOGARITHMS AND STATISTICAL DATA OF THE NUMBER OF CULTURABLE STAPHYLOCOCCI PER ML OF KIDNEY IN CONTROL MICE AND IN MICE TREATED WITH NEOMYCIN. CHALLENGE DOSE 1.8×10^7 BACTERIA

Day	Control		Neomycin		
	Mean	s ¹	Mean	s ¹	P ² <
1	5.05	1.11	2.33	0.24	0.001
3	5.06	1.81	0.29	0.64	0.001
5	6.40	3.58	0.57	0.81	0.01
7	4.85	3.94	3.47	4.09	—
10	1.94	3.77	1.56	3.50	—
12	3.95	3.01	0	0	0.05

¹ standard deviation

² probability that the mean is not different from that of the controls; given only when < 0.05

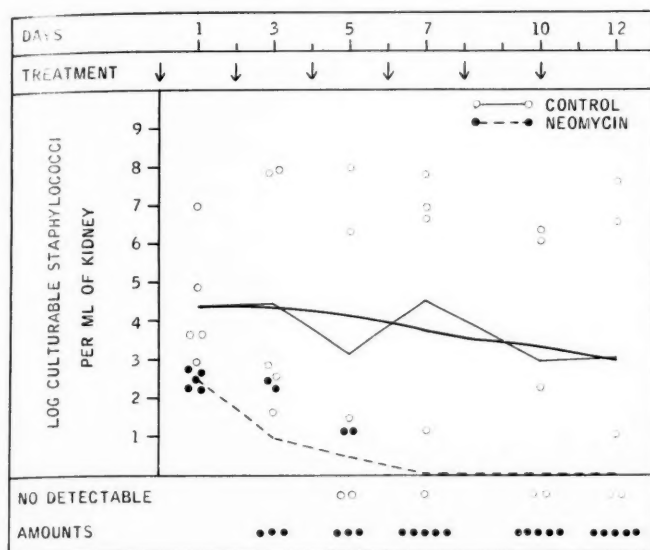


Fig. 2. — Diagram showing the logarithms of the number of culturable staphylococci per ml of kidney in control mice and in mice treated with neomycin (experiment 2). See also Table 2 and legend to Fig. 1.

TABLE 2

SECOND EXPERIMENT. LOGARITHMS AND STATISTICAL DATA OF THE NUMBER OF CULTURABLE STAPHYLOCOCCI PER ML OF KIDNEY IN CONTROL MICE AND IN MICE TREATED WITH NEOMYCIN. CHALLENGE DOSE 1.8×10^7 BACTERIA

Day	Control		Neomycin		
	Mean	s ¹	Mean	s ¹	P ² <
1	4.40	1.60	2.46	0.64	0.05
3	4.55	3.07	0.93	1.28	0.05
5	3.15	3.75	0.45	0.61	—
7	4.51	3.65	0	0	0.05
10	2.93	3.12	0	0	0.05
12	3.05	3.73	0	0	—

¹ standard deviation

² probability that the mean is not different from that of the controls; given only when < 0.05

after the fifth post-challenge day no bacteria were found in the kidneys of the treated animals.

DISCUSSION

The study was restricted to the kidneys which have been shown to be the most suitable organ for evaluating antibiotic agents *in vivo* by the microbial enumeration method (2). The present study showed the antibiotic effect of neomycin on experimental staphylococcal infection in mice in the same way as the effect of penicillin and ristocetin has been studied (2). Although neomycin can in general be applied only locally in clinical medicine, its antibiotic activity when administered subcutaneously could be evaluated by the enumeration method.

The relatively small bacterial dose used in the inoculum made it somewhat difficult to achieve homogenous results; the control group, too, had some animals with no detectable organisms in their kidneys from the fifth post-challenge day on. Despite this, the differences between the antibiotic and the control groups were statistically significant on most of the days on which determinations were made.

According to Waksman, Frankel and Graessle, 9–40 γ of neomycin/mouse was effective against *Staphylococcus aureus* infection induced intraperitoneally (4). Kiser and de Mello, who gave 800 γ of neomycin/mouse, attained 80–100 per cent protection (1). In our study the selected dose, 325 γ of neomycin/mouse per day, cured the infection rapidly. The results, however, cannot be directly compared, because of differences in bacterial and mouse strains and methods used.

SUMMARY

Neomycin, given subcutaneously to mice infected with *Staphylococcus aureus*, showed a high antibiotic activity evaluated by the microbial enumeration method from the kidneys.

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ADSORPTION ONTO AND ELUTION FROM ERYTHROCYTES OF THE TICK-BORNE ENCEPHALITIS VIRUS

by

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Since the discovery of the hemagglutinating property of arthropod-borne viruses by Sabin in 1950, the hemagglutination and hemagglutination-inhibition methods have been extensively employed in the study of the arbor viruses, especially in the serological identification of virus strains as well as in the demonstration of antibodies. The particular conditions required for the hemagglutination of arbor viruses are now well known. Little attention has been paid, however, to the mechanism of the hemagglutination phenomenon of these viruses.

In their studies of hemagglutination with some members of the arbor group, Sabin and Buescher (7) and Sabin (6) came to the conclusion that the combination ($H_x R_y$) between hemagglutinin (H_x) and the receptors (R_y) on red cells was of the equilibrium type, which may be expressed as $H_x + R_y \rightleftharpoons H_x R_y$. They stated also that the absence of hemagglutination outside of the required pH zone was due to failure of combination of the hemagglutinin with the erythrocytes. Chanock and Sabin (1) could not observe, however, a dissociation of the combined hemagglutinin outside the agglutination zone.

In addition to the studies with hemagglutinins, Dulbecco and Vogt (3) found that 80—90 per cent of the western equine encephalitis virus was adsorbed by a monolayer of susceptible cells

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within 30 minutes. Mims (4), also, reported that 75 per cent of the inoculated virus disappeared from blood within half an hour in experimental infections of mice with the Rift Valley fever virus.

In the present study experiments were conducted to find out the principles governing the adsorption of the diphasic tick-borne encephalitis virus onto red blood cells and to find out the means by which the adsorbed virus can be eluted from the erythrocytes. Since the stability of the tick-borne encephalitis virus hemagglutinin is very inconstant depending on pH and temperature (9), the experiments were based on infectivity which was assayed by the intracerebral technique in mice. The obtained results showed that the adsorption and elution of the tick-borne encephalitis virus completely differ from the other viral adsorptions so far known.

METHODS

Virus. — The experiments were carried out with the strain «Belyanchikov» of the Russian diphasic tick-borne encephalitis virus, isolated by Smorodintsev (10) from a patient near Leningrad in 1949. Since 1958 the strain has been used for experimental studies in our laboratory.

A homogenate from infectious suckling mouse brains was used as the source of virus in the adsorption experiments. The homogenate was prepared in the same manner as alkaline aqueous suspensions for studies of hemagglutination. The detailed technique of the preparation of the brain homogenate was as follows.

Suckling mice were inoculated intracerebrally at the age of 3—4 days with a dose of 10,000 LD₅₀. On the fifth day after the inoculation, when the mice were beginning to die, the brains were harvested. The mice were anesthetized with ether and partially exsanguinated by cutting open the thorax. The brains were removed and ground with quartz sand in a mortar. A 10 per cent suspension of brain tissue was made with a solution of 0.05 M borate—0.12 M NaCl—0.4 per cent bovine plasma albumin, pH 9.3. The brain suspension was centrifuged at 11,000 g for 60 minutes. The pooled supernatant fluids were stored in rubber stoppered tubes at -60°C until used.

In all the experiments reported here the same brain homogenate was used as the source of virus. The infectivity of the homogenate remained stable during the time of carrying out the experiments, which was about five months. The homogenate showed a hemagglutinating capacity up to a brain dilution of 1/25,600, pH 6.5, at + 4°C with 0.20 per cent rooster erythrocytes.

Since it was assumed that the brain homogenate in spite of its excellent hemagglutinating capacity may also contain unspecific hemagglutination inhibitors which might have an influence on adsorption, the amount of

possible inhibitors was determined in the following way. The hemagglutinin in the brain homogenate was first destroyed by incubating a sample at $+56^{\circ}\text{C}$ for 30 minutes, which completely inactivates the hemagglutinin without having any effect on the inhibitors. The hemagglutination-inhibiting activity of the heated sample was then assayed according to the standard technique (2) by using as antigen the same homogenate without heating.

Erythrocytes. — It was shown recently in this laboratory that rooster erythrocytes are agglutinable by the hemagglutinin of the tick-borne encephalitis virus, while adult hen erythrocytes usually are negative or give only poor results (8). Rooster erythrocytes were adopted for routine use in hemagglutination studies with the tick-borne encephalitis virus in our laboratory. Also the adsorption experiments in this study were carried out with rooster erythrocytes. In addition, the adsorbing capacity of goose erythrocytes was proved.

Erythrocytes were taken from the wing vein or by cardiac puncture into a syringe containing a sodium citrate-citric acid-dextrose solution as anticoagulant. The erythrocytes were washed five times with 10 volumes of a dextrose-gelatin-veronal solution.

Adsorption and Elution Experiments. — The adsorption fluid was composed of equal volumes of two solutions, *i.e.*, 0.05 M sodium borate—0.12 M NaCl—0.4 per cent bovine plasma albumin, pH 9.0, and 0.20 M sodium phosphate—0.15 M NaCl. By mixing different proportions of monobasic and dibasic sodium phosphates it was possible to obtain, after addition of the phosphate solution to an equal volume of borate-saline-albumin, the final pH values of 5.8—7.4. The albumin was necessary for the stabilization of the virus. The same solutions have been used by Clarke and Casals (2) for arbor virus hemagglutination studies.

The virus and erythrocyte concentrations for adsorption were chosen so that the adsorption fluid as a rule contained 10^8 live virus particles and the same number of erythrocytes per milliliter. This was approximately achieved by using in the adsorption a final brain dilution of 1:100 and a 5—10 per cent final erythrocyte concentration. The total volume of the adsorption fluid was generally 2.5 ml.

Unless otherwise stated in the tables or in the text, the pH of the adsorption fluid was 6.5 and adsorption took place for 20 minutes in a glass tube that was kept in an ice-water bath, with occasional shaking. After adsorption the erythrocytes were centrifuged at 1,500 r.p.m. for 5 minutes at 0°C . A sample for infectivity assay was usually taken from the supernatant. The rest of the supernatant was discarded and the erythrocytes were washed three times with 40 volumes of the same chilled solution as used for the adsorption. After the third washing the erythrocytes were suspended in borate-saline-albumin, pH 9.0, to the original volume of adsorption and the tube was placed in the ice-water bath for 20 minutes in order to produce elution. The erythrocytes were centrifuged and the supernatant contained the eluted virus.

Samples for infectivity assay were taken from the supernatants after

adsorption, from the eluates, and occasionally also from the erythrocyte washing solutions. The pH of the samples was adjusted to 9.0 by diluting 1:10 with borate-saline-albumin, pH 9.3, or by 1 M NaOH. The samples were stored at -60°C until assayed.

Infectivity Assays in Mice. — Samples to be tested for infectivity were diluted in serial tenfold dilutions with 0.05 M sodium borate — 0.12 M NaCl — 0.4 per cent bovine plasma albumin, pH 9.0. The tubes were kept in the ice-water bath while dilutions and inoculations were being made.

Young adult white laboratory mice weighing 10–12 gm. were employed in the infectivity assays. The mice were inoculated intracerebrally with a dose of 0.03 ml. Each dilution was injected into 5, or sometimes 10, mice. The inoculations were performed without anesthesia and without sterilization of the site of injection. The borate-saline-albumin solution, pH 9.0, was well tolerated by mice intracerebrally.

The mice were followed up for 12 days after inoculation and the LD_{50} endpoint was calculated from deaths according to the method described by Reed and Muench (5). However, deaths that occurred during the first three days after inoculation were regarded as being due to cerebral trauma and therefore were not taken into account. The total number of discarded mice was very small, being only 9 of the 1,420 inoculated mice equivalent to 0.6 per cent.

The reliability of the assay method was studied from parallel assays on the suckling mouse brain homogenate. The dilutions were made separately in the different assays. The standard deviation of the obtained LD_{50} values in logs was calculated from the formula:

$$s = \sqrt{\frac{\sum (x - \bar{x})^2}{n-1}}$$

where x is an individual LD_{50} value, \bar{x} is the arithmetic mean of the LD_{50} values, and n is the number of parallel assays. The smallest statistically significant difference between two individual LD_{50} values was obtained from the formula $t_{n-1} \times \sqrt{2} \times \text{standard deviation}$. The parallel assays were made by using only 5 mice per dilution and the standard deviation for 10 mice per dilution was not determined.

RESULTS

The reproducibility of the assay method was determined from 10 parallel assays on the suckling mouse brain homogenate that was used as the source of the virus in all the experiments described in this report. The parallel assays were performed on different days during a period of five months. The results of the different assays are presented in Table 1. The mean infectivity titer expressed as \log_{10} of LD_{50} was 8.43. The standard deviation of the results was 0.125. Based on this, a difference of $2.262 \times \sqrt{2} \times 0.125 = 0.4 \log$

TABLE 1

PARALLEL INFECTIVITY ASSAYS OF MOUSE BRAIN HOMOGENATE USED IN THE ADSORPTION AND ELUTION EXPERIMENTS

Number of Assay	Infectivity Assay of Brain Homogenate in Mice					LD ₅₀ in Logs
	10 ⁻⁵ *	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	
1	—	—	5/5**	3/5	0/5	8.2
2	—	—	5/5	3/5	1/5	8.3
3	—	5/5	5/5	4/5	0/5	8.4
4	—	4/4	5/5	4/5	0/5	8.4
5	—	—	5/5	4/5	0/5	8.4
6	—	—	4/5	4/5	1/5	8.4
7	5/5	5/5	5/5	5/5	0/5	8.5
8	—	—	5/5	4/5	1/5	8.5
9	—	—	4/5	5/5	1/5	8.5
10	—	5/5	5/5	4/5	2/5	8.7

* Dilutions of brain material.

** Numerator = number of deaths. Denominator = total number of mice.

between two individual LD₅₀ values was calculated to be significant with 95 per cent probability when 5 mice per dilution were used.

The adsorption of the tick-borne encephalitis virus onto erythrocytes was found to be primarily dependent on the pH of the adsorption fluid. In the experiment reported in Table 2 the adsorption was performed at different pH levels between 5.8 and 9.0 in the ice-water bath. In this experiment the washing of erythrocytes was exceptionally made 5 times with 40 volumes. The amounts of virus eluted from the erythrocyte samples were assayed. It appeared that the eluates of the adsorption pH's 6.2–6.8 contained 1,000–10,000 times as much virus as the eluates outside this adsorption pH

TABLE 2

EFFECT OF ADSORPTION PH ON THE AMOUNT OF ELUTED VIRUS

Adsorption pH	Infectivity Assay of Eluates in Mice								LD ₅₀ in Logs
	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	
pH 5.8	5/5	2/5	0/5	—	—	—	—	—	2.8
pH 6.2	—	—	—	5/5	4/5	2/5	0/5	0/5	6.7
pH 6.5	—	—	—	5/5	5/5	4/5	1/5	0/5	7.5
pH 6.8	—	—	—	5/5	4/5	0/5	1/5	0/5	6.5
pH 7.4	5/5	5/5	0/5	—	—	—	—	—	3.5
pH 9.0	4/4	4/5	1/5	—	—	—	—	—	3.5

range. The LD_{50} values of the latter eluates were, however, somewhat higher than the mathematically calculated dilution of the original virus after washing without specific adsorption. Since it is difficult to evaluate exactly the effectiveness of the washing, there is good reason to assume on the basis of the results that specific adsorption had occurred only in the range of pH 6.2–6.8, the maximum being at pH 6.5.

The amount of adsorbed virus was greatly dependent also on the temperature of the adsorption fluid. As seen in Table 3, the amount of eluted virus was 25 times greater when the adsorption had been carried out at 0°C than at + 37°C.

TABLE 3
EFFECT OF ADSORPTION TEMPERATURE ON THE AMOUNT OF ELUTED VIRUS

Adsorption pH and Temperature	Infectivity Assay of Eluates in Mice					LD_{50} in Logs
	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	
pH 6.5, 0°C	10/10	10/10	8/10	3/10	0/10	7.6
pH 6.5, + 37°C	10/10	5/10	2/10	0/10	0/10	6.2

The effect of the virus multiplicity on adsorption was studied by adding different amounts of erythrocytes to the constant concentration of virus, which was 10⁸ infective virus particles per milliliter. The experiment reported in Table 4 resulted in a significantly lower titer of eluted virus when the adsorption fluid contained five infective virus particles per one erythrocyte, as compared with the results obtained with a single virus particle or less per erythrocyte. Since there are reasons to believe that a noninfective virus behaves like an infective one in adsorption and elution, the proportion of noninfective viruses in the brain material may be of importance at high virus multiplicities. In the present study the ratio of infective and noninfective viruses was not determined.

TABLE 4
EFFECT OF VIRUS MULTIPLICITY IN THE ADSORPTION ON THE AMOUNT OF ELUTED VIRUS

Infective Virus: Erythrocyte Ratio in Adsorption Fluid with Constant Virus Titer	Infectivity Assay of Eluates in Mice					LD_{50} in Logs
	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	
1 : 0.2	5/5	5/5	2/5	0/5	0/5	6.8
1 : 1	5/5	5/5	4/5	1/5	0/5	7.5
1 : 5	5/5	5/5	4/5	2/5	0/5	7.7

When the pH of the erythrocyte suspension was changed beyond the pH range where adsorption occurs, at least to the alkaline side, the virus-erythrocyte complex was dissociated and the adsorbed virus was released into the fluid. As seen in Table 5, the elution of virus from erythrocytes was of the same degree at pH 7.4 as at pH 9.0 and independent of the temperature, whether 0°C or + 37°C.

TABLE 5

EFFECT OF PH AND TEMPERATURE OF ELUTION ON THE AMOUNT OF ELUTED VIRUS

Adsorption pH and Tem- perature	Elution pH and Tem- perature	Infectivity Assay of Eluates in Mice					LD ₅₀ in Logs
		10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	
pH 6.5, 0°C	pH 9.0, + 37°C	5/5	5/5	4/5	1/5	0/5	7.5
	pH 9.0, 0°C	5/5	5/5	5/5	0/5	1/5	7.6
	pH 7.4, 0°C	5/5	5/5	3/5	1/5	0/5	7.3

Experiments performed on the reaction velocity of adsorption and elution in Table 6 revealed that adsorption was completed within 15 minutes and elution already within 5 minutes. Accordingly, elution obviously takes place faster than adsorption.

TABLE 6

EFFECT OF TIME OF ADSORPTION AND ELUTION ON THE AMOUNT OF ELUTED VIRUS

Time of Adsorption	Time of Elution	Infectivity Assay of Eluates in Mice				LD ₅₀ in Logs
		10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	
5 min.	60 min.	5/5	3/5	0/5	0/5	7.2
15 min.	60 min.	5/5	4/5	2/5	0/5	7.7
60 min.	60 min.	5/5	5/5	2/5	0/5	7.8
60 min.	5 min.	5/5	5/5	1/5	0/5	7.6
	60 min.	5/5	5/5	2/5	0/5	7.8

Infectivity assays on supernatant fluids after adsorption resulted always in a high titer of remaining virus. It appears from Table 7 that the titer of the virus in the adsorption fluid decreased from 10^{8.5} before adsorption to 10^{8.1} after adsorption, showing that only 60 per cent of the virus was adsorbed onto the erythrocytes. The titers obtained in assays of eluates varied in the range 10^{7.8}—10^{7.5}, which is 1/4—1/8 of the expected value. The disappearance of the virus was assumed to be due to various reasons; for instance, a part of the adsorbed virus may be liberated during washings,

TABLE 7
PROPORTION OF ADSORBED VIRUS UNDER OPTIMAL CONDITIONS

Sample Tested	Infectivity Assay of Samples in Mice				LD ₅₀ in Logs
	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	
Virus before adsorption	10/10	10/10	9/10	1/10	8.5
Supernatant after adsorption	10/10	9/10	6/10	0/10	8.1
Eluate from erythrocytes	10/10	10/10	3/10	0/10	7.7

some of it may become inactivated during the procedure, or perhaps the elution is not complete.

Therefore, an experiment was made to study the effect of washings on the amount of virus adsorbed on erythrocytes, by washing an erythrocyte sample after adsorption 12 times with 10 volumes of fluid for 15 minutes each time. A part of the erythrocytes were taken for elution after the 3rd, 6th, 9th, and 12th washing. The results in Table 8 show that only a slight decrease, if any, could be found in the titers of eluted virus. Determinations of the virus titers of washing solutions confirmed the above result. The titers of washing solutions presented in Table 9 show that a small amount of virus actually was liberated from erythrocytes during the

TABLE 8
EFFECT OF REPEATED WASHING OF ERYTHROCYTES ON THE AMOUNT OF ELUTED VIRUS

Elution Carried out after	Infectivity Assay of Eluates in Mice					LD ₅₀ in Logs
	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	
3rd washing	—	5/5	5/5	2/5	0/5	7.8
6th washing	—	5/5	3/5	2/5	0/5	7.5
9th washing	5/5	4/4	5/5	1/5	—	7.6
12th washing	5/5	5/5	4/5	0/5	—	7.4

TABLE 9
LIBERATION OF ADSORBED VIRUS FROM ERYTHROCYTES INTO WASHING SOLUTIONS

Number of Washing Solution	Infectivity Assay of Washing Solutions in Mice					LD ₅₀ in Logs
	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	
3rd	—	5/5	5/5	4/5	0/5	5.4
6th	—	5/5	1/5	1/5	0/5	3.7
9th	4/4	4/5	0/5	0/5	—	3.4

TABLE 10
DEPENDENCE OF THE PROPORTION OF ADSORBED VIRUS ON THE CONCENTRATION OF VIRUS IN THE ADSORPTION FLUID

Amount of virus in Adsorption Fluid before Adsorption (LD ₅₀ per 0.03ml.)	Virus: Erythrocyte Ratio in Adsorption Fluid	Infectivity Assay of Supernatants in Mice							LD ₅₀ in Logs	Proportion of Adsorbed Virus (per cent)
10 ⁰ *	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸		
107.1	—	—	—	—	5/5	5/5	2/5	0/5	6.8	50 %
106.1	—	—	—	—	5/5	5/5	1/5	0/5	5.6	68 %
105.1	—	—	5/5	5/5	5/5	0/5	—	—	4.5	75 %
104.1	—	5/5	5/5	5/5	1/4	0/4	—	—	3.7	60 %
103.1	5/5	5/5	4/5	2/5	0/5	—	—	—	2.7	60 %
102.1	5/5	4/5	2/5	0/5	—	—	—	—	1.7	60 %

* Dilutions of the supernatant.

TABLE 11
ADSORPTION OF VIRUS FROM THE SAME SOLUTION BY NEW ERYTHROCYTE SAMPLES

Sample Tested	Infectivity Assay of Samples in Mice									LD ₅₀ in Logs
	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹			
Virus before adsorption	—	—	5/5	5/5	5/5	5/5	0/5	0/5	8.5	
Eluate of 1st adsorption	—	—	—	5/5	4/5	2/5	0/5	0/5	7.7	
Eluate of 2nd adsorption	—	—	5/5	5/5	3/5	0/5	—	—	7.2	
Eluate of 3rd adsorption	—	—	5/5	5/5	2/4	1/4	—	—	7.2	
Eluate of 4th adsorption	—	5/5	4/5	3/5	0/5	—	—	—	6.0	
Eluate of 6th adsorption	—	5/5	4/4	1/5	1/5	—	—	—	5.7	
Eluate of 10th adsorption	5/5	4/5	2/5	0/5	0/5	—	—	—	4.7	
Supernatant after 1st Adsorption	—	—	—	—	5/5	3/5	0/5	0/5	8.2	
Supernatant after 3rd Adsorption	—	—	—	5/5	5/5	1/5	1/5	1/5	7.7	
Supernatant after 10th Adsorption	—	—	5/5	5/5	4/5	1/5	—	—	7.5	

washings. The amount of liberated virus was, however, not more than 1/1,000—1/10,000 of the total virus present on erythrocytes. The experiment proved that it was impossible to wash off an essential amount of adsorbed virus from erythrocytes by lowering the virus content of the surrounding fluid.

The effect of the virus concentration on the proportion of adsorbed virus was studied by adsorbing serial tenfold dilutions of the brain homogenate by the 10 per cent erythrocyte concentration for 60 minutes. After the adsorption the supernatants were assayed for remaning virus. The amount of adsorbed virus was calculated by substracting the number of remaining virus from that of virus added. As seen in Table 10, about 60 per cent of the total virus was adsorbed in every case, regardless of the different multiplicities of virus which varied over a large scale up to a ratio of one virus per 25,000 erythrocytes.

Table 11 presents the results of 10 successive adsorptions on the same solution of virus with new erythrocyte samples for 15 minutes each time. The titers of virus eluted from the different samples of erythrocytes were gradually diminished from $10^{7.7}$ in the first adsorption to $10^{4.7}$ in the tenth adsorption. After the last adsorption the solution still contained virus up to a titer of $10^{7.5}$, showing that 1/10 of the original virus was left (Table 11.).

This unexpected result was explained after it was found that the brain material contained unspecific hemagglutination inhibitors that obviously act as soluble receptors in the adsorption. The amount of the inhibitors was 1/4—1/8 of the hemagglutinin present. The effect of the unspecific inhibitors on adsorption may be of quantitative importance only. This conclusion was suggested by a repeated adsorption of an earlier adsorbed and eluted virus. The eluate, which presumably did not contain inhibitors, was adsorbed also partially, although somewhat more completely. The results of the experiment are shown in Table 12.

Erythrocytes were found to be capable of adsorbing the virus at least two times successively, as is shown in Table 13. This confirms the finding of Sabin *et al.* (7, 6, 1) that no receptor-destroying enzyme is associated with the elution of the arbor viruses.

The experiments reported above were carried out with rooster erythrocytes. Also goose erythrocytes adsorbed the virus in the same manner as rooster erythrocytes, as is seen in Table 14.

TABLE 12

PROPORTION OF VIRUS ADSORBED IN REPEATED ADSORPTION OF AN EARLIER ADSORBED AND ELUTED VIRUS

Sample Tested	Infectivity Assay of Samples in Mice					LD ₅₀ in Logs
	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	
Earlier adsorbed virus used in the repeated adsorption	5/5	5/5	5/5	2/5	0/5	7.8
Supernatant after the repeated adsorption	5/5	5/5	2/5	1/5	0/5	7.0
Eluted of the repeated adsorption	5/5	5/5	2/5	0/5	0/5	6.8

TABLE 13

CAPACITY OF ERYTHROCYTES FOR REPEATED ADSORPTION OF VIRUS

Repeated Adsorption and Elution with the same Erythrocyte Sample	Infectivity Assay of Eluates in Mice					LD ₅₀ in Logs
	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	
1st adsorption and elution ...	5/5	5/5	4/5	1/5	0/5	7.5
2nd adsorption and elution ...	5/5	5/5	5/5	2/5	0/5	7.8

TABLE 14

COMPARISON OF THE ADSORBING CAPACITY OF ROOSTER AND GOOSE ERYTHROCYTES UNDER OPTIMAL CONDITIONS

Erythrocytes Used for Adsorption	Infectivity Assay of Eluates in Mice					LD ₅₀ in Logs
	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	
Rooster	5/5	5/5	4/5	1/5	0/5	7.5
Goose	5/5	4/5	5/5	2/5	0/5	7.7

DISCUSSION

The experiments described have shown the main principles governing the adsorption of the tick-borne encephalitis virus onto erythrocytes and the elution of the virus from them. It was found that the adsorption of the virus took place in the same pH range, between 6.2 and 6.8, where hemagglutination occurs and the virus adsorbed on erythrocytes was eluted from the erythrocytes outside this pH range. Adsorption was greatly influenced by the temperature, being 25 times greater at 0°C than at +37°C. Elution outside of the adsorption pH zone was independent of the temperature and occurred more rapidly than adsorption. After elution the ability of the erythrocytes to adsorb virus remained unchanged.

Some of the results obtained, however, were most likely affected by the unspecific hemagglutination inhibitors present in the brain material. Results of this kind were the relatively small proportion of adsorbed virus even under optimal conditions (Table 7), the removal of only 9/10 of the virus from a solution by successive adsorptions (Table 11), and possibly also independence of the proportion of adsorbed virus from the virus concentration (Table 10).

After the completion of the present series, adsorption and elution experiments were continued with the tick-borne encephalitis virus by using as the source of virus a sucrose-acetone extracted brain material (2) that did not contain inhibitors. The results so far obtained by using this kind of virus held true of the above findings with the only exception that 98 per cent of the virus was adsorbed from the acetone-extracted brain solutions, as compared to 60 per cent of the virus without extraction of brain tissue.

Further studies are still necessary to ascertain whether the adsorption reaction is of the true equilibrium type, and if so, to determine the equilibrium constant. Also the question of the number of virus particles that can be combined with one erythrocyte remains open.

The results obtained have shown that the adsorption of the tick-borne encephalitis virus is of a unique character, differing in its pH and temperature dependence from the known erythrocyte adsorptions of other virus groups. The adsorption described here has some resemblance in principle to the primary attachment of bacteriophages to the surface of the bacterium, which is primarily dependent on the cationic concentration but independent of the pH over a wide range.

The fundamental factors affecting the adsorption of the tick-borne encephalitis virus were the hydrogen ion concentration and the temperature. The role of pH can be understood, for instance, from the point of view that the net charge of proteins varies according to the hydrogen ion concentration. As is known, proteins possess an excess of positively charged amino groups on the acidic side and an excess of negatively charged carboxyl groups on the basic side of the isoelectric point. If the virus and the receptor on red blood cells have their isoelectric points at different pH levels, one of them is positively and the other negatively charged between these pH values, as a result of which electrostatic attraction occurs.

Consequently, the reaction between the virus and the receptor might be a salt linkage of proteins, where basic ($-\text{NH}_3^+$) groups of one side chain are united to acidic ($-\text{COO}^-$) groups of another chain. The influence of temperature on adsorption may be explained by the thermal movement acting against the attractive force.

Attempts to clarify the mechanism of the adsorption of arbor viruses on the basis of the above hypothesis are in progress.

SUMMARY

Experiments have been carried out to discover the principles governing the adsorption of the diphasic tick-borne encephalitis virus onto erythrocytes and the elution of the virus from the erythrocytes. The results were based on infectivity assays in mice. The reliability of the method was determined by parallel assays.

It was found that adsorption takes place in the same pH range, between 6.2 and 6.8, where hemagglutination occurs and that the virus-erythrocyte complex is dissociated outside of this pH range. Adsorption is greatly influenced by the temperature, being best in the cold. Elution, on the other hand, is independent of the temperature and occurs more rapidly than adsorption. After elution the ability of the erythrocytes to adsorb the virus remained unchanged.

Adsorption was partial. The ratio of adsorbed virus was diminished by unspecific hemagglutination inhibitors that were present in the brain material used as the source of virus. The unspecific inhibitors in the solution obviously act like the erythrocyte receptors.

The hypothesis concerning the mechanism of the adsorption is presented that adsorption occurs between the isoelectric points of the virus and the receptor, the reaction thus being an electrostatic salt linkage between ionized amino and carboxyl groups.

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REMOVAL OF TICK-BORNE ENCEPHALITIS VIRUS RECEPTORS FROM ERYTHROCYTES BY EXTRACTION WITH LIPID SOLVENTS

by

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It is known from the studies of Sabin and his associates (7, 6, 2) that normal human and animal serums contain unspecific inhibitors for the hemagglutinins of arbor viruses. These unspecific inhibitors probably are of the nature of lipids or lipoproteins, since they are thermostable, unaffected by periodate and proteases, and can be removed from serums by extraction with various lipid solvents.

Several methods have been suggested and employed for the elimination of the unspecific hemagglutination inhibitors from serums, viz., extraction with acetone (7, 6, 2), filtration through Seitz pads (1), adsorption with bentonite (3), and adsorption with kaolin (4). Kaolin adsorption is now in use as the standard treatment of serums before examination for hemagglutination-inhibiting antibodies.

The type of erythrocytes is of importance for the arbor virus hemagglutination. Erythrocytes of one-day-old chicks (7, 6), adult geese (5), and roosters (8) have proved suitable for studies of hemagglutination and hemagglutination inhibition, although erythrocytes from some other species of animal have also shown varying degrees of hemagglutinating capacity.

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This report shows that it is possible to extract from erythrocytes with fat solvents a substance strongly inhibiting the hemagglutination caused by the diphasic tick-borne encephalitis virus. Since the capacity of erythrocytes to adsorb the virus was remarkably reduced after the extraction, the conclusion was drawn that the extracted fraction probably contained most of the erythrocyte receptors for the tick-borne encephalitis virus.

MATERIAL AND METHODS

Erythrocytes. — Most of the experiments were carried out with rooster erythrocytes. In addition, red blood cells of one-day-old chicks, adult geese, and man were used for extraction. Blood was drawn into a citric acid-sodium citrate-dextrose solution and the cells were washed five times with 10 volumes of physiological saline.

Extraction with Lipid Solvents. — The solvents used were methanol, ethanol, chloroform-methanol 3:1, ethanol-ether 3:1, acetone, chloroform, light petroleum, and ether. Packed erythrocytes were the object of extraction. The extraction was carried out at room temperature three times successively, with 20 volumes of solvent and for 20 minutes each time. During extraction the erythrocyte-solvent mixture was shaken vigorously every fifth minute. The solvent was separated by centrifuging at 1,500 r.p.m. for 5 minutes. The solvents from successive extractions of the same erythrocyte sample were pooled and the pool was once more centrifuged until clear. The solvent was evaporated in vacuum at 40—50°C. The dry residue obtained was suspended by means of a glass rod in borate-saline-albumin, pH 9.0, to make 10 volumes of the original packed erythrocytes. The resulting milky suspension was subjected to the hemagglutination inhibition test of the diphasic tick-borne encephalitis virus.

After extractions with acetone or ethanol-ether, the erythrocytes were resuspended in saline by using the glass rod. The erythrocytes were washed 5—10 times with saline in order to obtain an evenly distributed and colorless suspension. The extracted erythrocytes were then used for adsorption experiments of the tick-borne encephalitis virus.

Technique of Hemagglutination Inhibition. — The standard method described by Clarke and Casals (4) was employed, with the following specification.

The virus used for the preparation of the hemagglutinating antigen was a Russian strain «Belyanchikov» of the diphasic tick-borne encephalitis virus, isolated by Smorodintsev from a patient near Leningrad in 1949. The antigen was prepared from infected mouse brains by using the sucrose-acetone extraction method. The extracts were studied without foregoing erythrocyte absorption. The borate-saline diluent contained also 0.4 per cent bovine plasma albumin. Serial twofold dilutions of the extracts were incubated overnight at 4°C with 4-8 units of the antigen. Rooster erythro-

cytes in a concentration of 0.20 per cent were used as indicator and the settling of erythrocytes took place at pH 6.5 in the refrigerator.

Adsorption of Infective Virus. — The principles of the adsorption onto and elution from erythrocytes of the tick-borne encephalitis virus have recently been discovered in our laboratory (10). By employing the method described by us earlier, the ability of erythrocytes to adsorb the virus before and after extraction was determined. The procedure was essentially as follows.

The adsorption was performed at 1–2°C, pH 6.5, for 20 minutes. The adsorption fluid contained 10^8 infective virus particles and the same number of erythrocytes per ml. After adsorption the erythrocytes were washed three times with 40 volumes of the same chilled solution. The elution of the virus from erythrocytes took place at pH 9.0 for 20 minutes. The eluates were assayed for infective virus by the intracerebral technic in young adult mice.

Adsorption of Hemagglutinin. — A comparison of the adsorbing capacity of erythrocytes before and after extraction was also made, based on the adsorption of the hemagglutinin of the tick-borne encephalitis virus. The method was in principle similar to the above. However, owing to the instability of the hemagglutinin on the acid side of neutrality (9), the procedure at pH 6.5 should be carried out as quickly as possible. For this reason the adsorption time was reduced to 5 minutes, the washing was done twice with 40 volumes, and only one tube was handled at a time. In this way the procedure at pH 6.5 did not exceed 20 minutes, during which time the hemagglutinin titer decreased to $1/2$ – $1/4$. Finally, the hemagglutinating activity of the eluates was determined.

RESULTS

Extracts obtained from packed erythrocytes with the usual fat solvents were capable of inhibiting the hemagglutination caused by the tick-borne encephalitis virus. The inhibiting activity of extracts depended on both the solvent and the erythrocytes used.

The capacity of various solvents to extract the inhibitory substance from rooster erythrocytes is presented in Table 1. It was found that the most effective solvents were methanol, ethanol chloroform-methanol, ethanol-ether, and acetone, whereas extraction with chloroform, light petroleum, and ether produced less active extracts. The inhibiting fraction could be isolated at least from chick, rooster, goose, and human erythrocytes (Table 2). Other erythrocytes have not been studied so far.

As seen in Table 1, many of the erythrocyte extracts agglutinated rooster erythrocytes in low dilutions without virus, after which

TABLE 1
HEMAGGLUTINATION-INHIBITING PROPERTIES OF EXTRACTS OBTAINED FROM ROOSTER ERYTHROCYTES WITH VARIOUS SOLVENTS

Solvent	Hemagglutination Inhibition Assay of Extracts (+ = hemagglutination; - = inhibition)										Inhibition Titer of Extract
	1/10*	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	
Methanol	+	+	-	-	-	-	-	-	+	+	1/1280
Ethanol	+	+	+	+	-	-	-	-	+	+	1/1280
Chloroform-methanol 3:1**	+	+	+	+	-	-	-	-	+	+	1/1280
Ethanol-ether 3:1	+	+	+	-	-	-	-	-	+	+	1/1280
Acetone	+	+	-	-	-	-	-	+	+	+	1/640
Chloroform	-	-	-	-	+	+	+	+	+	+	1/80
Light petroleum	+	-	-	-	+	+	+	+	+	+	1/80
Ether	-	-	-	+	+	+	+	+	+	+	1/40

* Dilutions are based on the volume of packed erythrocytes.

** Volumetric proportions.

TABLE 2
HEMAGGLUTINATION-INHIBITING ACTIVITIES OF EXTRACTS OBTAINED WITH
ACETONE FROM VARIOUS KINDS OF ERYTHROCYTES

Origin of Erythrocytes	Inhibition Titer of Acetone Extract
Chick (one day old)	1/1280
Rooster	1/640
Goose	1/640
Man	1/320

only inhibition became visible. The panagglutinin titers of different extracts varied in the range of 1 : 10—1 : 80.

In extraction with chloroform-methanol, chloroform, light petroleum, and ether, the erythrocytes were converted into a more or less amorphous mass. After extraction with the other solvents the erythrocytes appeared to be in a fair condition morphologically. They were, however, agglutinable spontaneously, for which reason they could not be used for hemagglutination studies. Nevertheless, the extracted erythrocytes were suitable for adsorption experiments.

The adsorption experiment reported in Table 3 revealed that the ability of rooster erythrocytes to adsorb the infective virus was reduced to 1/16 and 1/50 after extractions with acetone and ethanol-ether, respectively. An analogous experiment with hemagglutinin reported in Table 4 showed a reduction to 1/8—1/16 in the adsorbing capacity of erythrocytes after extraction with acetone. The adsorp-

TABLE 3
CAPACITY OF ROOSTER ERYTHROCYTES FOR ADSORPTION OF THE INFECTIVE VIRUS
BEFORE AND AFTER EXTRACTIONS WITH ACETONE AND ETHANOL-ETHER

Erythrocytes Used for Adsorption of Infective Virus*	Infectivity Assay of Eluates in Mice						LD ₅₀ of Eluates (in Logs)
	10 ⁻⁴ **	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	
Unextracted erythrocytes	—	5/5***	5/5	3/5	2/5	0/5	7.5
Acetone extracted erythrocytes	5/5	5/5	3/5	1/4	0/5	—	6.3
Ethanol-ether extracted erythrocytes	4/4	4/4	2/5	0/5	0/5	—	5.8

* Titer of the virus used 10^{-8.5} LD₅₀ per 0.03 ml.

** Dilutions of brain tissue.

*** Numerator = number of deaths; denominator = total number of mice.

TABLE 4
CAPACITY OF ROOSTER ERYTHROCYTES FOR ADSORPTION OF THE HEMAGGLUTININ
BEFORE AND AFTER EXTRACTION WITH ACETONE

Number of Experiment	Erythrocytes Used for Adsorption of Hemagglutinin *	Hemagglutination Assay of Eluates							Hemagglutination Titer of Eluate
		1/100**	1/200	1/400	1/800	1/1600	1/3200	1/6400	
I	Unextracted erythrocytes	+	+	+	+	+	—	—	1.1
	Acetone extracted erythrocytes	+	+	—	—	—	—	—	1.2
II	Unextracted erythrocytes	+	+	+	+	+	+	—	1.3
	Acetone extracted erythrocytes	+	+	+	—	—	—	—	1.4
III	Unextracted erythrocytes	+	+	+	+	+	+	—	1.3
	Acetone extracted erythrocytes	+	+	—	—	—	—	—	1.2

* Titer of the hemagglutinin used 1/25,600.

** Dilutions of brain tissue.

tion experiments thus indicated that the extraction had removed from the erythrocytes most of the receptors for the tick-borne encephalitis virus.

COMMENT

The results have shown the presence in erythrocytes of an inhibitor for the arbor virus hemagglutination. It is probable that the inhibitor substance is responsible for the specific adsorption of the arbor viruses onto erythrocytes. The similar solubility characteristics of the serum inhibitor and the erythrocyte receptor support the opinion that they are identical. Further studies are necessary on the physical properties and chemical nature of the inhibitor and receptor substances before the results can be related to other known facts.

SUMMARY

It was found to be possible to extract from erythrocytes with lipid solvents a fraction strongly inhibiting the hemagglutination caused by the diphasic tick-borne encephalitis virus. The most effective solvents were methanol, ethanol, chloroform-methanol, ethanol-ether, and acetone, whereas chloroform, light petroleum, and ether were less effective. The active fraction could be isolated from all the erythrocytes studied, *e.g.*, from chick, rooster, goose, and human erythrocytes.

After extraction the ability of erythrocytes to adsorb the infective virus or the hemagglutinin of the tick-borne encephalitis virus was reduced to $1/8$ — $1/50$. It seems probable that the extraction had removed most of the erythrocyte receptors for the tick-borne encephalitis virus.

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MITOTIC ACTIVITY IN THE EPITHELIUM OF THE ESOPHAGUS, TRACHEA AND SMALL BRONCHI OF TWO- AND THREE-MONTH OLD MICE¹

by

HARRY BJÖRK and MATTI HÄRKÖNEN

(Received for publication February 8, 1960)

In a earlier article in this journal (1957:35:223) one of us (H.B.) described the mitotic rate and colchicine sensitivity of the epithelium of the esophagus, trachea and bronchi of the white mouse. Two-month old mice were used in that study.

In another study published in this journal (1960:38:110) we compared the mitotic activity in the epithelium of the small bronchi in 3-month old mice with (A/Jax) and without (C57) a tendency to develop spontaneous lung tumour. The two strains were not found to differ as regards normal mitotic activity or the response to colchicine.

The present investigation was undertaken in an attempt to compare the mitotic activity in the epithelium of the esophagus, trachea and small bronchi of mice aged two and three months. For that purpose the results of the above studies were supplemented with mitotic counts on esophageal and tracheal epithelium in the 3-month old mice used for the latter of the above two studies.

The mouse is not considered adult until the age of three months. It seemed of interest to determine the normal mitotic frequency and the response to colchicine in pre-adult and adult mice, i.e. at the ages of two and three months respectively.

The series consists of 89 mice, on which mitotic counts were

¹ This investigation was supported by the Damon Runyon Memorial Fund (291—D).

performed from the epithelium of the esophagus, trachea and small bronchi. Thirty of these were 2-month old albino mice of unspecified strain, 30 were 3-month old albino mice of A/Jax strain, and 29 were 3-month old gray mice of C57 strain.

The animals of each strain were divided into two groups. One was studied without using carioclastics, the other after subcutaneous injection of 0.09 mg of colchicine. The animals were weighed. They were decapitated in batches of five, the times of exposure to colchicine being 5 hours, 11—12 hours, and 29 hours. The untreated animals were also killed in batches at the same times of the day. The specimens were fixed in Bouin's solution. The slides were stained with hematoxylin-eosin, and the number of mitoses was calculated per 3000 cells.

RESULTS

Surprisingly enough, the mean weight of the 2-month old mice was somewhat higher (22.3 gm.) than that of the 3-month old mice (A/Jax 21.0 gm., C57 21.8 gm.).

Table 1 illustrates the normal average frequency of mitoses per 1000 cells in the epithelium of the organs examined in the three strains of mice.

TABLE 1

AVERAGE FREQUENCY OF MITOSES PER 1000 EPITHELIAL CELLS OF THE ESOPHAGUS, TRACHEA AND BRONCHI IN UNTREATED 2- AND 3-MONTH OLD MICE

Age and Strain	Number of Animals	Average Frequency of Mitoses		
		Esophagus	Trachea	Bronchi
2 months, albino	15	4.6	1.0	2.5
3 months				
A/Jax, albino ..	15	3.8	0.19	0.29
C57, gray	15	3.2	0.12	0.55

Naturally, the highest mitotic count occurred in the esophagus in the case of all three strains. The next highest counts were found in the bronchi, the lowest in the trachea.

A small and not significant difference in the mitotic rate in the esophagus was observed between the 2-month old (4.5) and the 3-month old mice (3.8 and 3.2). In the other organs, however, there was a distinct difference: for the trachea the mitotic counts in

the 2-month old mice were five and eight times as high, and for the bronchi eight and five times as high as in the two series of 3-month old mice ($P < 0.01$ in all these comparisons).

The response to colchicine is shown in table 2.

TABLE 2
AVERAGE FREQUENCY OF MITOSES PER 1000 EPITHELIAL CELLS OF THE
ESOPHAGUS, TRACHEA AND BRONCHI IN COLCHICINE-TREATED (0.09 MG) 2- AND
3-MONTH OLD MICE

Age, Strain, Organ	Number of Animals	Average Frequency per Group	Average Frequency per Subgroup		
<i>2 months, albino</i>	15		a)	b)	c)
Esophagus		33.9	17.8	36.2	47.9
Trachea		14.9	15.3	15.8	13.7
Bronchi		7.53	1.46	14.3	7.14
<i>3 months</i>					
<i>A/Jax, albino</i>	15				
Esophagus		20.4	11.4	18.2	31.7
Trachea		1.12	0.78	1.44	1.14
Bronchi		1.01	0.74	1.38	0.92
<i>C57, gray</i>	14				
Esophagus		20.9	13.4	17.6 ¹	31.0
Trachea		1.81	1.24	2.93 ¹	1.42
Bronchi		1.03	1.00	1.65 ¹	0.56

a) Killed 3—4 p.m. (exposure to colchicine 5 hours)

b) " 9—11 p.m. (" " " 11—12 hours)

c) " 3—4 p.m. (" " " 29 hours).

¹ This subgroup consists of 4 animals, all others of 5 animals.

The figures for the two strains examined at the age of three months showed close agreement. The similarity in response to colchicine in the small bronchi, referred to in our previous communication, was thus found to apply also to the esophagus and the trachea.

As in the untreated animals, the mitotic rate in the esophagus was higher after colchicine injection in the 2-month old (33.9) than in the older mice (20.4 and 20.9). However, even here the difference is not statistically significant. The trachea and the bronchi, on the other hand, show a much more distinct difference between the two age groups, as did the untreated animals. The average obtained for the trachea in the 2-month old mice was higher by thirteen and eight times respectively than in the 3-month old mice

(<P0.001 in both comparisons), and with regard to the bronchi the figures were in both cases eight times as high in the younger as in the older animals ($P < 0.05$ and $P = 0.02$).

The mode of response to colchicine was essentially similar in the three strains. The mitotic rate in the esophagus showed a progressive increase during the course of the experiment: it was highest in the last count, performed 29 hours after injection. In the trachea and the bronchi the state of affairs was different: with no single exception the highest count was obtained 11–12 hours after injection.

DISCUSSION AND CONCLUSIONS

The great similarity, also in absolute terms, between the mitotic rates obtained for the 3-month old albino A/Jax mice and the gray C57 mice suggests that both the normal mitotic rate and the response to colchicine in the esophagus, trachea and bronchi are similar in young mice of different strains at the same age.

The difference between the mitotic rates obtained in the esophagus of 2- and 3-month old mice is slight. In the trachea and bronchi, however, the mitotic rates of both the untreated animals and those treated with colchicine are clearly higher in the 2-month old than in the 3-month old mice. This is undoubtedly due to the fact that the squamous epithelium in the esophagus during the whole life span is the site of active regeneration owing to the continuous desquamation of cells. The low mitotic rates in the trachea and bronchi of adult mice indicates that the epithelial regeneration in these organs as compared with the esophagus is negligible after the individual has reached adulthood.

The mode of response to colchicine in the esophageal squamous epithelium differs from that seen in the respiratory epithelium of the trachea and bronchi. The mitotic frequency increases continuously in the esophagus from one test to the next during the observation time of 29 hours, whereas the response in the trachea and bronchi already subsides during the latter half of the observation time. This must also be regarded as an expression of the higher mitotic activity in the esophagus as compared with the corresponding biological phenomenon in the trachea and the bronchi.

SUMMARY

Thirty 2-month old mice (albino, unspecified strain) and 59 3-month old mice (albino A/Jax and gray C57) were studied with respect to the normal mitotic frequency and the colchicine response in the epithelium of the esophagus, trachea and the small bronchi.

The difference between the mitotic rates obtained from the esophagus of untreated and colchicine-treated animals of different ages was relatively slight. In the tracheal and bronchial epithelium many times as high mitotic rates were obtained for the 2-month old untreated and colchicine-treated mice as for the 3-month old mice. This is attributed to the active regeneration of the epithelium of the esophagus during the entire life span, whereas in the respiratory epithelium regeneration is relatively slight in adult life.

The response to colchicine, given as a dose of 0.09 mg., continues for a considerably longer time in the esophagus than in the respiratory epithelium, which phenomenon is considered a further sign of the higher mitotic activity in the squamous epithelium.

RAPID DETERMINATION OF UREA

by

O. M. FORSELL and I. P. PALVA

(Received for publication February 6, 1960)

A method of determining urea on the basis of the reaction between diacetylmonoxime and urea has been applied to clinical use by Andersen, Friedman and Rosenthal. The principal disadvantages of the technique have been the lability of the colour formed and the dependance of its intensity on the reaction time. To overcome these disadvantages Marsh *et al.* went as far as to use automatic equipment. Interesting in its simplicity is Wearne's statement that this disadvantage can be overcome by the use of a pressure cooker. We tested its serviceability especially for routine determinations in our laboratory.

METHOD

Reagents. (1) NaOH 1/16 N; (2) ZnSO₄ 10 per cent; (3) Arsenic acid 0.265 N in hydrochloric acid; (4) Diacetylmonoxime (Fluka) 2.5 per cent.

Procedure. To one volume of serum are added 8 volumes of 1/16 N NaOH and 1 volume of 10 per cent ZnSO₄ to precipitate the protein. The precipitate is removed by centrifuging. To 0.5 ml of clear filtrate is added 2 ml of distilled water, 1.5 ml of 0.265 N arsenic acid in hydrochloric acid and 0.5 ml of 2.5 per cent diacetylmonoxime. The solution is mixed well and the volume is made up to 5 ml with distilled water. The tube is closed loosely with a glass stopper moistened in the reaction mixture by tilting the tube.

The samples are placed in a domestic pressure cooker of stainless steel and kept at 2 atm. pressure for 15 minutes. They are cooled under a running tap and the extinction measured in 10 mm cuvettes at a wave length of 478 m μ using a Beckman B spectrophotometer.

DISCUSSION

The relationship between extinction and urea concentration is shown in Fig. 1. It will be seen that the extinction is directly proportional to the urea concentration up to 80 mg of urea nitrogen in

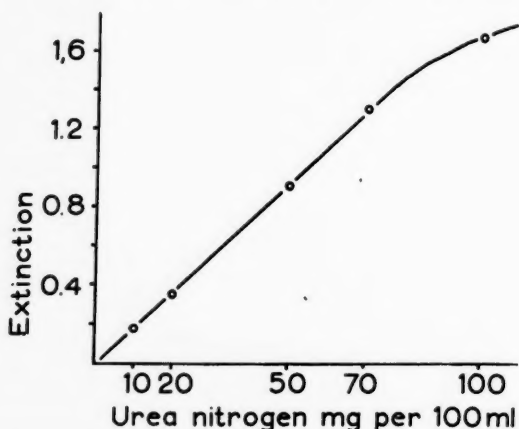


Fig. 1. — Relationship between extinction and urea nitrogen concentration.

100 ml. This is in agreement with the results of Rosenthal who studied the diacetylmonoxime method in detail. The curves plotted by Friedman and Andersen assume the shape of an S also at the lower ends, and this obviously causes uncertainty in the determination of small concentrations.

Fig. 2 shows the effect of the reaction time on the result obtained. On heating in an open waterbath the intensity of the colour increases even after 60 minutes, although very little. On heating in a pressure cooker it takes 15 minutes to reach the level from which there is no subsequent change in colour intensity, as Wearne reported. The reaction is thus complete at raised pressure although the reaction time may not always be exactly the same, and it is consequently not necessary to provide standards with each determination.

Andersen and Friedman report that the colouring in the reaction

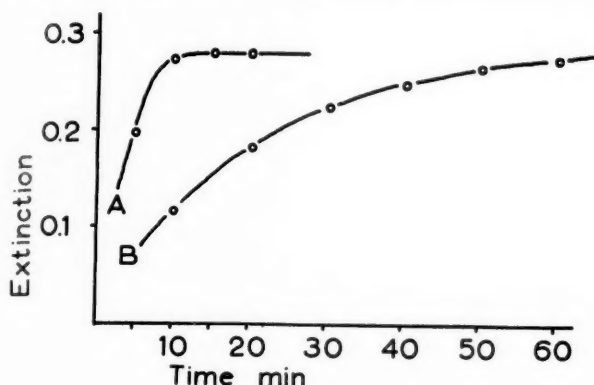


Fig. 2. — Relationship between period of heating a 15 mg per 100 ml urea nitrogen solution and extinction A: in a pressure cooker, B: in an open water bath.

disappears on exposure to light. The colour forming under pressure seems to be more stable. According to Wearne, only 3 per cent of the colour disappears in 1 hour in fluorescent light and 12 per cent on standing for two days. No change big enough to affect the result was observed by us in 6 hours in ordinary laboratory lighting.

We also tested urea in Folin-Wus tungstate filtrate, but because of precipitation did not obtain reproducible values.

SUMMARY

It is possible to make the determination of urea based on diacetylmonoxime more rapid and stable by performing the reaction under pressure. An ordinary domestic pressure cooker suffices. The reaction is always complete and the colour formed is stable to light. The procedure is simple, rapid and reliable and as such suitable for routine use.

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AGGLUTINATION BY RHEUMATOID ARTHRITIC SERA
OF SHEEP RED CELLS SENSITIZED WITH
NORMAL CATTLE SERA

by

ANJA TIILIKAINEN and O. MÄKELÄ¹

(Received for publication February 16, 1960)

Many rheumatoid arthritic sera are capable of agglutinating sheep erythrocytes sensitized by a subagglutinating dose of anti-sheep cell hemolysin, while most normal human sera are not (6, 8, 9). Sensitized with the homologous antibodies, the cells also of other species can be used in the hemagglutination test (4, 9, 11); and the rheumatoid arthritic factor (RAF) is generally believed to be common (1), to most of the cases at least, *viz.* the «agglutination activating factor» of Waaler (8). On the contrary, none of the normal sera has sensitized the cells to the rheumatoid arthritic factor in spite of the fact that anti-sheep cell agglutinins occur in them (9). This has given rise to the belief that RAF reacts with immune globulins only, or with a complex of immune antibody and antigen, in the same way as complement does (10). The aim of this paper is to show that normal sera, too, may be capable of rendering sheep's erythrocytes sensitive to the action of RAF; also, that the capability is lost when the normally occurring anti-sheep cell agglutinins of the serum are absorbed with a small amount of untreated sheep cells.

¹ Aided by a grant from the Sigrid Jusélius Foundation.

MATERIAL AND METHODS

Erythrocytes. — Sheep and human blood was collected, with citric acid—dextrose solution as anticoagulant. The blood was stored at $+4^{\circ}\text{C}$ for not more than 4 days. The cells were washed three times immediately before use.

Sera Used for Sensitization. — Sera from normal adult people, guinea pigs, horses, cows, and rabbits, or rabbits immunized with sheep red cells, were heated (at 56°C for 30 minutes) after withdrawal of the blood, and they were stored at -20°C .

Sera Used for Agglutination. — These were inactivated as above, and anti-sheep red cell agglutinins were absorbed with half the volume of packed cells. Sera from normal adult people and from rheumatoid arthritic patients were used.

A 0.9 per cent NaCl solution was used as diluent.

Trypsin Treatment of Red Cells: One volume of washed and packed sheep red cells was mixed with four volumes of Sørensen's phosphate buffer, pH 7.7, containing 0.1 per cent of crystalline trypsin (Trypure, Novo). After being treated at 37°C for 30 minutes the cells were centrifuged down and washed once.

Sensitization of Red Cells: Normal human, guinea pig, horse, bovine, and rabbit sera and rabbit anti-sheep sera were used for the sensitization of sheep red cells. Equal volumes of the serum dilution (containing $\frac{1}{2}$ — $\frac{1}{4}$ of the minimum agglutinating dose if not otherwise stated) and 0.5 per cent red cell suspension were mixed. After two hours' incubation at room temperature the cells were centrifuged down, the supernate was decanted and enough saline added to make a 0.5 per cent suspension. The sensitization of human red cells with anti-Rh₀ (D) was carried out as has been described earlier (5).

Agglutination Tests. Of the sera to be examined serial twofold dilutions were made in bulk. Of these dilutions, 0.05 ml amounts were distributed into round-bottomed tubes with a Pasteur pipette. Next, 0.05 ml of the 0.5 per cent suspension of red cells (normal or sensitized) was pipetted into the tubes. After two hours incubation at room temperature the results were read with the naked eye.

RESULTS

The present work was prompted by the accidental discovery that certain bovine sera were able to sensitize sheep's erythrocytes so as to make the cells agglutinable by some rheumatoid arthritic sera but not by normal human sera (the anti-sheep agglutinins of the human sera were removed by absorption before the experiment).

Ability of different normal sera to sensitize sheep cells.

It will be seen from Table I that sheep cells sensitized by subagglutinating dilutions of different normal rabbit sera in one case are agglutinated by a selected rheumatoid arthritic serum. The sensitizing power of normal bovine sera varies but is in all cases lower than that of the anti-sheep erythrocyte sera tested. An observation not apparent from Table I is the loss by the most powerfully sensi-

TABLE I
ABILITY OF DIFFERENT NORMAL SERA, AND RABBIT ANTI-SHEEP HEMOLYSIN SERA TO SENSITIZE SHEEP ERYTHROCYTES SO AS TO RENDER THE CELLS AGGLUTINABLE BY A RHEUMATOID ARTHRITIC SERUM

Titer of the Rheumatoid Arthritic Serum	Normal sera					Rabbit Anti-sheep Erythrocyte Sera
	Bovine	Rabbit	Guinea Pig	Horse	Human	
1: <4	25	12	10	3	5	
1: 4	9					
1: 8	9					
1: 16	11					
1: 32	14					
1: 64	2					
1: 128	2					
1: 256		1				
1: 512						
1: 1024						1
1: 2048						
1: 4096						1
Total	72	13	10	3	5	3

tizing bovine sera of that power when the anti-sheep erythrocyte agglutinins were absorbed by normal sheep red cells from the sera.

A study was made of the ability of some bovine sera to agglutinate sheep's cells treated with trypsin. In Table II the ability of the working dilutions ($= \frac{1}{2}$ agglutinating dose) to render sheep cells

TABLE II

CORRELATION BETWEEN THE «SENSITIZING» POWER AND ABILITY OF DIFFERENT NORMAL CATTLE SERA TO AGGLUTINATE TRYPSIN-TREATED SHEEP RED CELLS

Ability to «Sensitize»	Ability to Agglutinate Trypsin-treated Cells	
	good ¹	Weak
good	7	2
weak	4	6

¹ This means that the dilution used for «sensitization» (which did not agglutinate untreated sheep cells) still caused an agglutination after a further 2 to 4 fold dilution. (The cattle sera had been chosen from both ends of the scale beforehand.

sensitive to the agglutinating action of rheumatoid arthritic sera was compared with their ability to agglutinate trypsin-treated sheep cells. No close correlation could be established.

Table III shows that, at best, 1/32 of the minimum agglutinating dose of the cow serum will suffice to bring about sensitization. The

TABLE III

CORRELATION BETWEEN THE AGGLUTINATION TITER OF A RHEUMATOID ARTHRITIC SERUM, AND THE «SENSITIZING» DOSE OF A RABBIT ANTI-SHEEP HEMOLYSIN AND A SELECTED NORMAL CATTLE SERUM

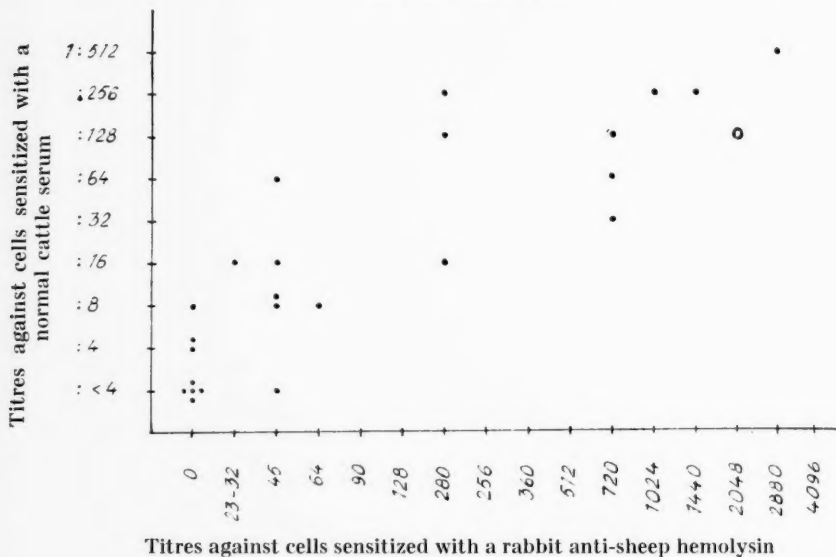
Sensitization Carried out with	Dilution of the Rheumatoid Arthritic Serum											
	1:2	:4	:8	:16	:32	:64	:128	:256	:512	:1024	:2048	:4096
Rabbit anti-sheep serum												
MAD ¹ : 2	+++	+++	++	++	++	++	+	+	+	(+)	(+)	—
MAD: 4	+++	+++	++	++	++	++	+	+	+	—		
MAD: 8	++	++	++	++	+	+	+	+	—			
MAD: 16	+	+	+	+	+	+	+	—?	—			
MAD: 32	(+)	—?	—									
MAD: 48	—?	—										
Cattle serum N:o 19												
MAD: 2	++	++	+	++	+	+	+	—				
MAD: 4	++	++	++	++	+	+	+	—				
MAD: 8	++	++	++	+	+	—?	—					
MAD: 16	++	++	+	+	—?	—						
MAD: 32	+	+	+	—								
MAD: 48	—?	—										

¹ Minimum Agglutinating Dose

TABLE IV
ABILITY OF DIFFERENT HUMAN SERA TO AGGLUTINATE SHEEP RED CELLS SENSITIZED WITH A NORMAL CATTLE SERUM

Agglutination Titter	N:o of Normal Human Sera	N:o of Rheumatoid Arthritic Sera
1: <4	34	14
1: 4	2	3
1: 8	3	2
1: 16	1	3
1: 32		6
1: 64		3
1: 128		3
1: 256		4
1: 512		2
1: 1024		0
Total of the sera	40	40

Fig. 1. — The agglutination titres of the different rheumatoid arthritic sera against sheep cells sensitized with a selected cattle serum or with a rabbit anti-sheep hemolysin.



○ this serum was able to agglutinate human O Rh positive erythrocytes sensitized with anti-Rh₀

rabbit anti-sheep sera appear to be almost equal to cow sera in sensitizing power.

Ability of different human sera to agglutinate sheep cells sensitized with normal cattle sera or with rabbit anti-sheep hemolysins, or human O Rh positive cells sensitized with a selected incomplete anti-Rh₀.

Table IV shows that, with few exceptions, only rheumatoid arthritic sera will agglutinate sheep's cells sensitized with bovine serum. Of rheumatoid arthritic sera, however, by no means all are capable of doing so. Fig. 1 shows the correlations of the agglutination titres of the different rheumatoid arthritic sera a) with sheep cells sensitized by rabbit anti-sheep antibodies, b) with sheep cells sensitized by bovine sera, and c) with human cells sensitized by anti-Rho. The correlations are not close, which suggests that the «rheumatoid arthritic factors» responsible for the three reactions are not fully identical.

DISCUSSION

It seems (on the basis of the results) that the factor in rheumatoid arthritic sera which is responsible for the agglutination of sheep erythrocytes «sensitized» with selected cattle sera is closely related to the RAF or «agglutination activating factor» of Waaler (8). This assumption is supported by the fact that the factors both affect red cells, which are coated with antibody-like substances, and that they both mainly occur in rheumatoid arthritic sera only.

As yet the «agglutination activating factor» has not been found to have an effect on such red cells as have been sensitized with normal heteroagglutinins or isoagglutinins (9).

It is not easy to explain why the heteroagglutinins and isoagglutinins of several species fail to render erythrocytes sensitive to the rheumatoid factor, while the corresponding immune agglutinins or normal heteroagglutinins of cattle are capable of doing so. The results that have been presented in the foregoing do not constitute definite evidence for the view that the incomplete anti-sheep agglutinins of cattle might be alone responsible for the sensitization. As the problem is not clarified data from experiments with rabbit anti-bovine globulin sera will be presented at a later stage.

On the basis of these results it seems possible that the sera of selected cattle immunized with sheep's erythrocytes might be of value in the study of RAF.

Those factors in rheumatoid arthritic serum which react differently with sensitized red cells do not seem to be identical, because the quantities of the various constituents in different rheumatoid sera are not always proportional. This observation is in harmony with what has been found out by Heller *et al.* (2,3), *viz.* that there are qualitative differences between sheep erythrocyte reactants and F II reactants of rheumatoid arthritic sera. Additional evidence for the rheumatoid factor being a complex of reactants will be presented in another paper (7).

SUMMARY

It was found that sheep erythrocytes treated with subagglutinating doses of certain normal cattle sera were agglutinated by several rheumatoid arthritic sera but not by normal human sera. The significance of the observation that also normal heteroagglutinins may be capable of rendering red cells sensitive to the action of the rheumatoid arthritic factor is discussed.

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THE ADVANTAGE OF INCUBATION IN HYDRAZOIC ACID CONTAINING AIR IN THE ENUMERATION OF FAECAL STREPTOCOCCI BY THE MEMBRANE FILTER TECHNIQUE

by

HELGE GYLLENBERG and SEPPO NIEMELÄ

(Received for publication February 16, 1960)

Slanetz and Bartley (3) have devised a selective medium for the enumeration of faecal streptococci, particularly for use in connection with the membrane filter technique. This medium contains, among other ingredients, sodium azide as the selective agent, and triphenyltetrazolium chloride (TTC) which gives rise to colored, and hence more easily countable colonies. The high selectivity of Slanetz' and Bartley's medium has been confirmed in this laboratory, but in several experiments a rather wide variation in the colorization, size and number of colonies was observed. As this may make the counts obtained less reliable, and a differentiation of subtypes on the basis of the color and size of the colonies definitely impossible, experiments concerning some details of the method were carried out in order to obtain a reduction of the variation described.

METHODS

Most filtration experiments were performed with a strain of human origin. As this strain tolerated sodium tellurite in a concentration of 1/2500 (w/v) and sodium chloride in a concentration of 6.5 per cent (w/v), and was able to ferment sorbitol, it was considered to be representative of *Streptococcus faecalis* (cf Shattock; 2; Spaander and Roest, 4). Two other cultures were also employed;

the one was type culture No. 7171 from National Collection of Type Cultures (London), named *Streptococcus faecium*, and the other a strain of equine origin (marked H 1). The last mentioned culture was characterized by its failure to tolerate sodium tellurite and sodium chloride.

For filtration experiments organisms from stock cultures (agar slants) were suspended in sterile water. Further dilution was performed to obtain a final suspension containing <10 organisms/ml. 10 to 20 ml of this suspension were used for each filtration, thus resulting in the development of <200 colonies (70 to 100 in most experiments) on each membrane. Filtration equipment as well as membranes from the Membranfilter-Gesellschaft, Sartoriuswerke A.G., Göttingen (West-Germany) were employed.

After filtration, the membranes were placed on agar in small Petri dishes (diameter 70 mm). The agar was composed according to Slanetz and Bartley (3), and when not stated otherwise it contained 0.04 per cent (w/v) of sodium azide. The medium was sterilized by ordinary autoclaving, and the TTC (which was sterilized separately by filtration) was added in connection with the pouring of plates. Incubation followed at 37°C for 44 to 46 hours. The colonies were counted with the aid of a stereo microscope (magnification 10x). The data presented in Tables 1 and 2 are means of the counts from 3 or 4 parallel plates.

In the experiments concerning the effect of a hydrazoic acid containing air, the plates were incubated according to Gerencser and Weaver (1) in a closed vessel (we used ordinary laboratory desiccators with volumes of about 5 000 ml) together with a separate beaker containing a mixture of 7.5 ml of 0.5 per cent (w/v) of sodium azide and 7.5 ml of 5 N hydrochlorid acid. When this procedure was employed, the sodium azide was left out from the medium.

RESULTS AND DISCUSSION

The aim of the topical method is reached when maximal colorization of the colonies is obtained under conditions which give rise to maximal numbers of colonies. It could be concluded already from preliminary experiments that the depth of the medium in the dish (i.e. the volume of agar used to pour each plate) caused

variation in both of these figures. More exact experiments showed that colony colorization and the numbers of colonies unfortunately are affected in opposite ways by differences in the medium depth. Increasing volumes of the sodium azide medium per plate cause increase in both the strength and frequency of colony colorization, but at those amounts of medium needed to obtain a 100 per cent colorization, a decrease in the colony numbers was observed (Table 1). This reduction in colony numbers at high agar depths is ob-

TABLE 1

EFFECT OF THE MEDIUM VOLUME PER PLATE ON THE COLONY NUMBERS AND COLORIZATION OF COLONIES UNDER DIFFERENT CONDITIONS OF INCUBATION. COLUMNS A: AVERAGE NUMBERS OF COLONIES PER MEMBRANE, COLUMNS B: AVERAGE PERCENTAGE OF TOTALLY COLORED COLONIES.

Volume of Medium per Plate, ml	<i>Experiment 1</i> No Sodium Azide, Normal Incubation		<i>Experiment 2</i> Sodium Azide in Medium		<i>Experiment 3</i> No Sodium Azide, Incubation in Hydrazoic Acid Atmosphere	
	A	B	A	B	A	B
2	102	0	67	0	83	0
4	97	5	81	5	82	6
6	84	14	62	34	77	29
8	83	32	87	47	75	63
10	90	100	47	100	75	100
12	84	100	26	100	80	100
14					79	100
15			10	100		
16					82	100

viously due to the sodium azide in the medium. As can be seen from Table 1, no corresponding drop in colony numbers was observed in experiments with Slanetz' and Bartley's medium without sodium azide. This conclusion led us to test the technique recently described by Gerencser and Weaver (1), which is based on the fact that the effect of sodium azide is due to its transformation to hydrazoic acid, and that this acid is volatilized and effectively re-absorbed into the medium from the air. The data given in Table 1, as well as the photographs presented on Plate 1 clearly show that this method makes it possible to overcome the partial inhibition of colony development observed when high volumes of sodium azide containing medium are used for selective enumeration of

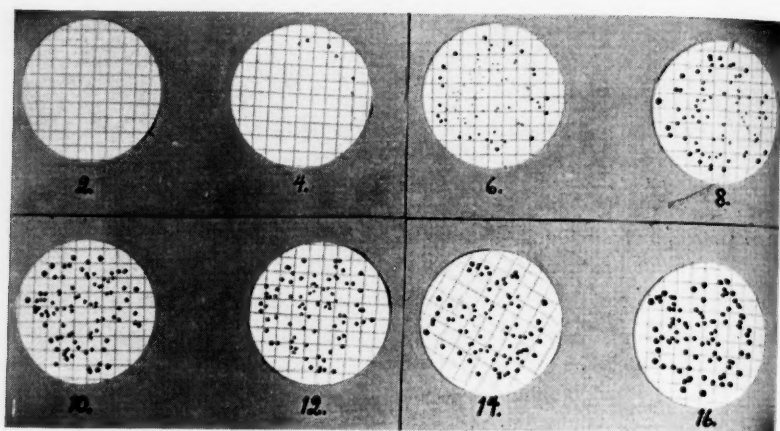
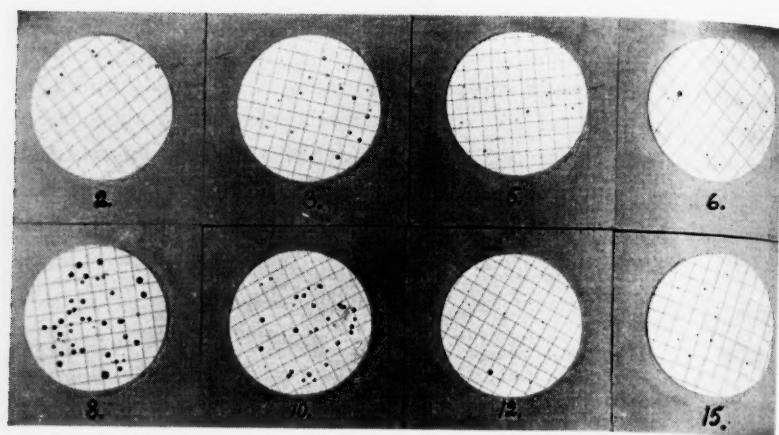


Plate 1. — Effect of the volume of medium per plate on the numbers and colorization of the colonies which develop on the membranes. A. Sodium azide is incorporated in the medium, incubation in ordinary atmosphere. B. No sodium azide in the medium, incubation in hydrazoic acid atmosphere. — Numbers indicate the volume of medium per plate.

faecal streptococci on membrane filters. As can be seen from Table 2, the hydrazoic acid atmosphere method may also give rise to a slight drop in colony numbers as compared with incubation without hydrazoic acid (or sodium azide), but this effect seems to be independent of the amount of medium in the plates.

TABLE 2

EFFECT OF HYDRAZOIC ACID ATMOSPHERE ON THE COLONY COUNTS WITH DIFFERENT TEST STRAINS. FIGURES REFER TO AVERAGE NUMBERS OF COLONIES PER MEMBRANE.

Test Strain	Incubation in	
	Ordinary Atmosphere	Hydrazoic Acid Atmosphere
<i>Streptococcus faecalis</i>	101	95
<i>Streptococcus faecium</i>	72	63
Strain H 1	37	31

No special experiments were conducted to show why sodium azide when incorporated in the medium becomes inhibitive in plates containing high volumes of medium. We suppose, however, that this is due to a much more effective reabsorption, and thus enrichment at the agar surface, of volatilized hydrazoic acid in plates containing much agar than in plates with small volumes of medium. Plates containing a high volume of sodium azide medium may give off more hydrazoic acid than plates with less medium, but as the air space in the first-mentioned plates is proportionally smaller, the difference in the hydrazoic acid concentration of the air in contact with the agar surface must be still more accentuated.

SUMMARY

Sodium azide is commonly incorporated as a selective agent in media used for the enumeration of faecal streptococci. When membrane filter techniques are employed also triphenyltetrazolium chloride is often added in order to obtain colorization of the colonies. It is shown in the present report that, with the membrane filter technique, a high volume of agar per plate supports the colorization of colonies, but reduces colony counts. To obtain maximal colony counts under conditions which give rise to maximal colony colorization, sodium azide must be left out from the medium. The selectivity of the method for faecal streptococci can be maintained, however, if the plates are incubated in a closed vessel together with a separate solution of sodium azide in hydrochloric acid. With this technique the volume of agar used to pour the plates does not influence colony counts or colony colorization.

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STABILITY OF MUMPS VIRUS AT $+35^{\circ}\text{C}$

by

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The heat-stability of the mumps virus has been studied mainly in view of the preparation and preservation of antigens and vaccines. Some experience has been gained about the inactivation rates of different viral properties at the temperatures available in virological laboratories (2, 13, 10, 12, 5, 1, 6). However, also rather divergent observations about the stability of mumps virus have been made in different laboratories (13, 6, 12).

When studying virus reproduction and problems associated with it, it is more important to know the stability of the virus at the temperature of multiplication than at any other temperature. As far as the author knows in the case of mumps virus there is only one study in which the inactivation rate of the infectivity at $+35^{\circ}\text{C}$ has been measured. According to this report (8) the half-life of infectivity of mumps virus in allantoic fluid at $+35^{\circ}\text{C}$ is about 80 minutes. In view of some earlier experiences in this laboratory this time looked surprisingly short. Therefore the present study was undertaken. Inactivation rates of the infectivity and hemagglutinins of several mumps strains at $+35^{\circ}\text{C}$ *in vitro* will be presented below.

MATERIAL AND METHOD

Viruses:

Enders strain was supplied in 1949 by Dr. Herbert R. Morgan. It had gone through an unknown number of allantoic passages. In

¹ Aided by a grant from the Sigrid Jusélius Foundation.

the experiments below the strain was in the 128th to the 133rd and in 168th allantoic passages in Finland.

Habel strain was obtained from Dr. Victor J. Cabasso in 1955 in the 23rd egg passage. After twelve amniotic passages in this laboratory the first attempt was made to pass the virus allantoically. The yield of hemagglutinins was irregular in the first few passages, but after about ten passages the allantoic fluids began to show hemagglutinins regularly. In the experiments below the strain was in the 71st to 76th allantoic passages.

KS strain was isolated by the author from the saliva of a mumps patient in January 1957. Not until the third amniotic passage was hemagglutination positive in the amniotic fluid. After eight amniotic passages the first attempt was made to pass the strain allantoically and since the first allantoic passage the strain has grown well in the allantoic cavity. The virus was identified as mumps virus by the hemagglutination inhibition test with anti-Enders fowl serum and by the complement fixation test with a known positive human convalescent serum. In the experiments below the 29th and the 33rd allantoic passages were employed.

HeWe strain was isolated by the author from the saliva of a mumps patient in February 1957. The amniotic fluid in the first passage already gave a positive hemagglutination. After four amniotic passages the strain has passed allantoically and it has grown well. The strain was identified as mumps virus in the same way as the KS strain. In the experiments below the 32nd and 36th allantoic passages were used.

The techniques employed in the *allantoic passages*, in the *hemagglutination titrations* and in the *infectivity titrations* have been described in full details earlier in this journal (4). Also the reproducibility of the infectivity and hemagglutination titrations were studied in the foregoing paper.

Inactivation experiments:

Pooled allantoic fluids derived from standard passages (inocula diluted 10^{-4} — 10^{-6} ; incubation time 5 days. (4) were placed immediately after harvesting at $+35^{\circ}\text{C}$ in rubber stoppered flasks. Samples were taken at intervals and the hemagglutination and/or infectivity titres were immediately determined.

RESULTS

Inactivation experiments with three mumps virus strains are illustrated in figure 1. It shows that the infectivity of all the strains decreased roughly at the same rate in view of the accuracy of the titration method (4). Table 1. gives the rate of decrease in infectivity

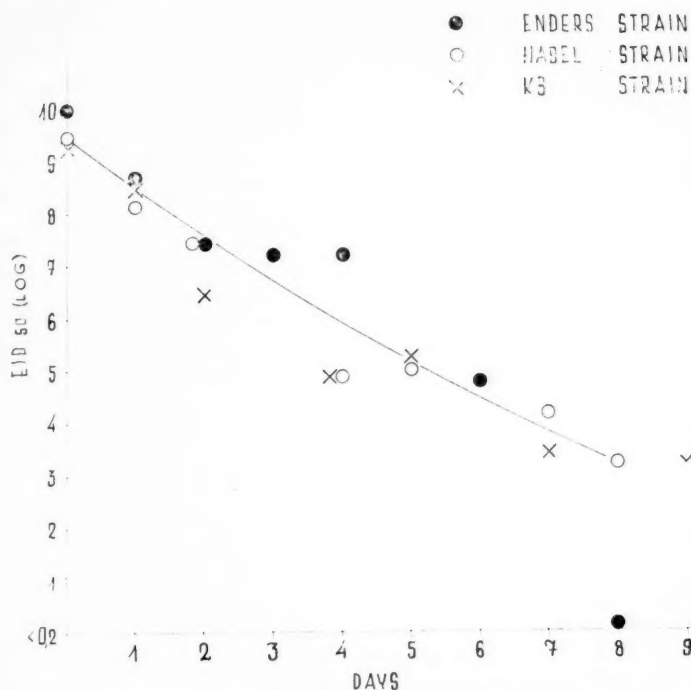


Fig. 1. — Inactivation rate of infectivity of different mumps strains at +35°C *in vitro*.

TABLE 1

ENDERS STRAIN. INACTIVATION RATE OF INFECTIVITY IN ALLANTOIC FLUID AT +35°C *IN VITRO*

Days	EID ₅₀ (log)		Decrease of EID ₅₀		Half-life (Hours)
	from	to	total	per day	
1	10.0	8.7	1.3	1.3	5.5
2	10.0	7.4	2.6	1.3	5.5
3	10.0	7.2	2.8	0.9	8.0
4	10.0	7.2	2.8	0.7	10.3
6	10.0	4.8	5.2	0.9	8.0
5.5	8.7	2.7	6.0	1.1	6.5

TABLE 2
HABEL STRAIN. INACTIVATION RATE OF INFECTIVITY IN ALLANTOIC FLUID
AT +35°C IN VITRO

Days	EID ₅₀ (log)		Decrease of EID ₅₀		Half-life (hours)
	from	to	total	per day	
1	9.5	8.1	1.4	1.4	5.1
2	9.5	7.5	2.0	1.0	7.2
4	9.5	4.9	4.6	1.2	6.0
5	9.5	5.0	4.5	0.9	8.0
7	9.5	4.2	5.3	0.8	9.0
8	8.4	3.2	5.2	0.7	10.3

TABLE 3
KS STRAIN. INACTIVATION RATE OF INFECTIVITY IN ALLANTOIC FLUID
AT +35°C IN VITRO

Days	EID ₅₀ (log)		Decrease of EID ₅₀		Half-life (hours)
	from	to	total	per day	
1	9.3	8.6	0.7	0.7	10.3
2	9.3	6.4	2.9	1.5	4.8
4	9.3	4.9	4.4	1.1	6.5
5	9.3	5.2	4.1	0.8	9.0
7	9.3	3.5	5.8	0.8	9.0
9	9.3	3.2	6.1	0.7	10.3

TABLE 4
STABILITY OF THE HEMAGGLUTININS OF ENDERS AND HABEL STRAINS IN
ALLANTOIC FLUIDS AT +35°C IN VITRO

Time of storage at + 35°C	Enders strain		Habel strain	
	HA titre (log)			
	from	to	from	to
7 days	2.55	2.4	2.85	1.5
7 "			2.7	2.55
7 "			2.7	1.8
10 "	2.7	2.85	2.7	2.25
10 "	2.1	2.25	2.55	0.9
10 "	3.0	3.0	2.4	1.8
14 "	3.0	3.0	1.8	<0.6
14 "			2.7	<0.6
8 weeks	2.55	1.35	2.7	<0.3
9 "	2.7	2.7	2.4	<0.3
4 months	2.7	1.8	2.55	<0.3

of the Enders strain calculated on the basis of these experiments. It can be seen that the infectivity dropped roughly 1 log per day. The half-life (= the time in which the number of infective doses will be reduced by one half, i.e. will decrease by 0.3 log) varied from 5.5 to 10.3 hours as calculated from the titration values of different inactivation days. The mean was 7.3 hours.

The half-life values of the infectivity of the Habel strain (table 2.) varied from 5.1 to 10.3 hours under the corresponding conditions. The mean was 7.7 hours. With the KS strain (table 3) these values were 4.8 to 10.3 hours respectively under similar conditions. The mean was 8.3 hours.

The pH of the virus allantoic fluids studied was measured. It was in every case between 7.5 to 8.0.

Some data on the stability *in vitro* at +35°C of the hemagglutinins of the Enders and Habel strains are collected in table 4. It shows that in none of the virus allantoic fluids of the Enders strain did the hemagglutinin titre drop significantly within 1 to 2 weeks. Even after storage of several months at +35°C large amounts of hemagglutinins were still present. The hemagglutinin of the Habel strain, however, appeared to be clearly more unstable at +35°C. The rate of reduction in the hemagglutination titres of different virus preparations differs considerably, but after 1 to 2 weeks the titres of most of the preparations already showed a distinct decrease and after some months the hemagglutinating activity was entirely lost.

Hemagglutinin titres of four mumps virus strains kept simultaneously *in vitro* at +35°C are given in figure 2. It shows that the strains studied fall into two groups as regards the stability of the hemagglutinin at +35°C. The Habel and KS strains completely lost the hemagglutinating activity within 3 to 4 weeks whereas the hemagglutinin titres of the Enders and the HeWe strains kept at the original level for at least 5 weeks. The lability of the hemagglutinin of the KS strain, compared with the Enders and HeWe strains, was confirmed by other experiments. However, it was seen, as earlier with the Habel strain, that different virus preparations of the same strain could give rather divergent results.

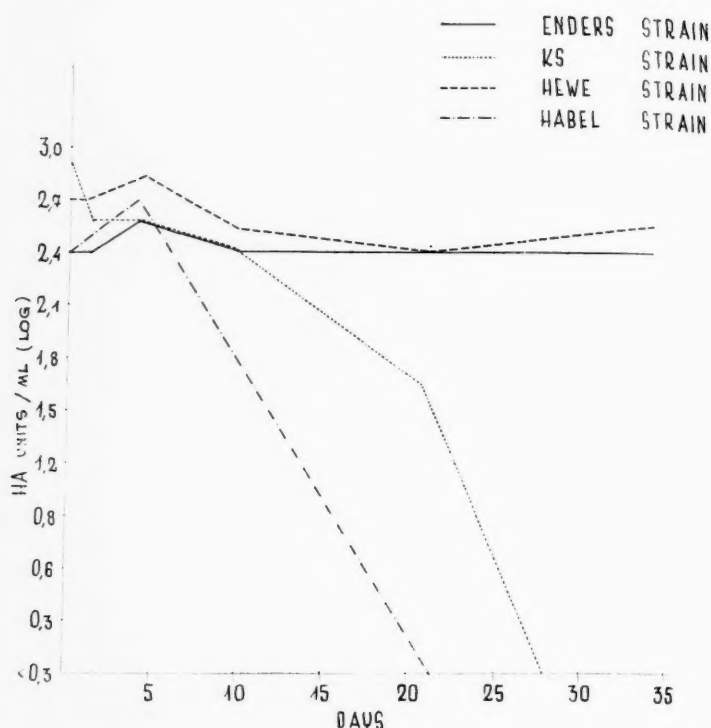


Fig. 2. — Inactivation rate of hemagglutinins of four mumps strains at +35°C *in vitro*.

DISCUSSION

According to Horsfall (8) the half-life of infectivity of mumps virus (the strain was not stated) in allantoic fluid at +35°C is about 80 minutes corresponding to a decrease in infectivity of 5.4 logs in a day. Thus, the infectivity of a virus preparation should be completely destroyed in less than two days. This is clearly inconsistent with the observations made in this work. The corresponding half-life value obtained in this study was 7 to 8 hours on an average and a total loss in infectivity was never seen in less than eight days. The easiest way to account for this discrepancy would be to assume that the heat-stability of the strains (or lines) used varies considerably. It is, however, conspicuous that also in the case of influenza PR₈ virus clearly lower half-life figures for the infectivity

at $+35-37^{\circ}\text{C}$ have been obtained by Horsfall (7) than by Paucker and Henle (11). This has been interpreted as meaning that the stability of different lines of the same strain can vary greatly (9). However, the methods employed by the different workers for the measuring of half-life have not been identical. Horsfall calculated the half-life from the values obtained during the first few hours of inactivation. Paucker and Henle (11) used the same method as in this study, *i.e.* the calculation of half-life from the values of several days' inactivation. It has been noticed, however, that the loss in infectivity of influenza PR₈ in undialyzed allantoic fluids at $+37^{\circ}\text{C}$ may be more pronounced during the first day of inactivation than during the following days (11). Might this perhaps be partly responsible for the low figures obtained by Horsfall? In any case, only parallel inactivation experiments with the different virus lines or strains can definitely show how much they really differ as regards heat-stability. The three strains studied above did not differ significantly in this respect.

It has been demonstrated earlier that the hemagglutinin of the Habel strain is more readily destroyed than that of the Enders strain at $+37^{\circ}\text{C}$ in the presence of 0.1 per cent of formalin (3). The results above show that the same fact is true also in the absence of formalin. Moreover, the two other strains studied differed in the same manner, the one having a labile hemagglutinin like that of the Habel strain, the other a stable hemagglutinin like that of the Enders strain. The heat-stability of the hemagglutinin might perhaps be used as a marker identifying different mumps virus strains.

SUMMARY

Inactivation at $+35^{\circ}\text{C}$ *in vitro* of the infectivity and the hemagglutinating activity of four mumps virus strains was studied. All the virus preparations were infected allantoic fluids; the pH was between 7.5 and 8.0.

The infectivity of all the strains studied (Enders, Habel and KS strains) dropped roughly 1 log per day. The half-life, when calculated from the titration values of different inactivation days, varied from 5 to 10 hours. The mean was 7 to 8 hours.

The Habel and KS strains completely lost the hemagglutinating

activity within 3 to 4 weeks, whereas the Enders and HeWe strains retained their hemagglutinating capacity for considerably longer times. The rate of decrease in hemagglutinin titres could vary greatly in different virus preparations of the same strain.

The reasons for contradictory results obtained in this and in an earlier study (8) are discussed.

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ROLE OF INHIBITORS IN HEMAGGLUTINATION BEHAVIOR OF MUMPS VIRUS

FURTHER STUDIES

by

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In an earlier paper from this laboratory (6) it was shown that the poor ability of some strains of mumps virus to agglutinate human red cells (10, 3, 17) is only apparent and caused by the contamination of virus preparations by inhibitors. The reduction of the amount of inhibitors in the virus preparations made these almost equally capable of agglutinating human red cells as chick red cells.

It has been reported that red cells of some animal species, *e.g.* cat (18), mouse (9, 8), and ground squirrel (1) are inagglutinable by mumps virus. The object of this paper is to study whether observations of that type are still tenable after a partial purification of virus preparations from inhibitors. In addition, the complement fixation and the hemagglutination tests are compared with respect to their ability to detect mumps virus in the presence of hemagglutination inhibitors.

MATERIAL AND METHODS

Viruses. — The histories of the Enders and the Habel strain used have been described in an earlier paper (4). Both the strains were passed allantoically by inoculating 0.2 ml of 10^{-4} diluted virus (about 10^{4-5} EID₅₀ per inoculum) into 7 to 8 days' eggs.

¹ Aided by a grant from the Sigrid Jusélius Foundation.

After incubation of five days at $+35^{\circ}\text{C}$ the allantoic fluids were harvested. All the virus preparations used in the experiments below were infected, pooled allantoic fluids.

Red Cells. — Red cells from the following species were used: Fowl, guinea pig, sheep, horse, cow, man, mouse and cat. The fowl cells used were previously tested and found sensitive to mumps virus. Human red cells were always taken from the same person belonging to blood group O. The mouse red cells were obtained from pooled blood of several mice: the red cells of the other species were taken from individual animals.

The blood was drawn from a suitable superficial vein or from the heart into a syringe containing 3.8 per cent sodium citrate solution. The red cells were washed three times and stored under sterile conditions in a 10 per cent suspension at $+4^{\circ}\text{C}$.

Hemagglutination Titrations. — Dilutions of the virus were made in bulk in phosphate buffered saline (pH 7.4) and 0.25 ml amounts were distributed on plastic plates to give triplicate dilution series for each type of red cells. The first dilution series was kept one hour at $+4^{\circ}\text{C}$, the second at room temperature (about 21°C) and the third at $+37^{\circ}\text{C}$. To the dilution series were added 0.25 ml of 0.5 per cent red cell suspension having the corresponding temperature. The results were read after two hours. The results are given as reciprocals of the last dilution giving 50 per cent agglutination.

Complement Fixation Titrations. — The technique employed in the CF titrations was exactly the same as in the earlier study, where it is described in detail (4). Only some essential points will be given here. The immune serum consisted of human mumps convalescent serum taken three weeks after the onset of disease. It was used in dilution 1 to 8 corresponding to 16 antibody units with an optimal dilution of crude mumps allantoic fluid antigen. 1.26 full units of complement (pooled fresh guinea pig serum) were employed. The fixation time was 18 hours and the temperature $+4^{\circ}\text{C}$.

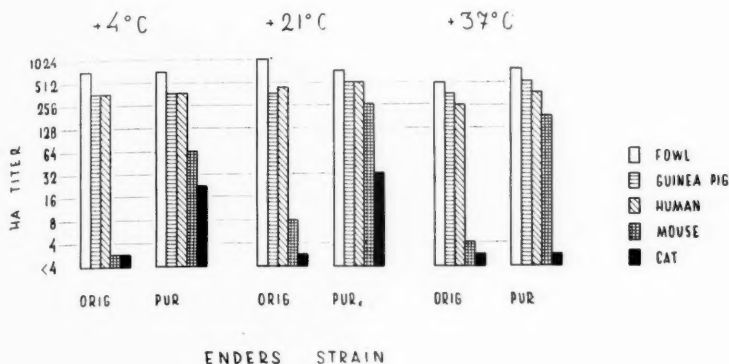
Partial Purification of the Virus. — 10 ml of virus allantoic fluid were cleared by centrifugation (Christ, type UJ₃) for 20 minutes at 3000 r.p.m. The supernatant was taken and ultracentrifuged (Spinco model L) for 45 minutes at 34,906–73,300 g. The supernatant was carefully removed and discarded and the sediment was made up to 10 ml of 1/15 M phosphate buffered

saline (pH 7.4). The same procedure of low speed—high speed centrifugation was repeated.

Ultrasonic Treatment.—5 ml of partially purified virus preparation was treated in MSE Mullard ultrasonic disintegrator (20 KC) for 30 minutes. The cooling was done with iced water.

RESULTS

Figure 1 shows that the original virus fluid of the Enders strain failed to agglutinate cat red cells at any temperature and that



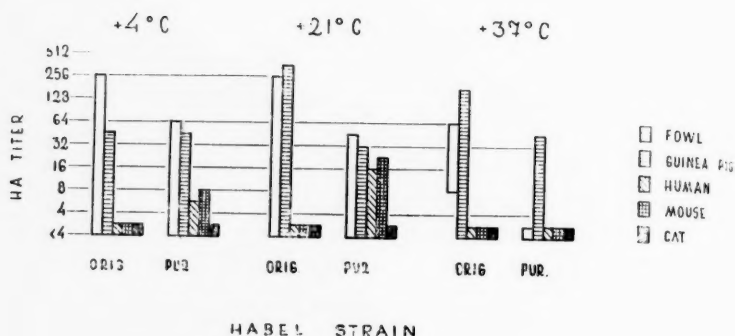
ORIG. = original virus fluid

PUR. = partially purified virus preparation

Fig. 1. — Effect of purification of mumps virus (Enders strain) on the capacity of the virus to agglutinate red cells of various species.

the mouse red cells were only agglutinated very weakly. These results are consistent with earlier observations (9, 8, 18). The partial purification of the virus preparation, however, clearly changed the reciprocal relations between the titers obtained with different red cells. The purification of the viruses did not essentially change the fowl, guinea pig and human titers, but definitely raised the mouse and cat titers. The mouse erythrocytes were agglutinated at all the temperatures; at +21°C and +37°C almost as well as fowl, guinea pig and human cells. Even with the purified virus the cat titers remained clearly inferior to the others and at +37°C the cat cells completely failed to agglutinate.

A similar experiment with the Habel strain is illustrated in figure 2. The original virus fluid of the Habel strain behaved in



ORIG. = original virus fluid

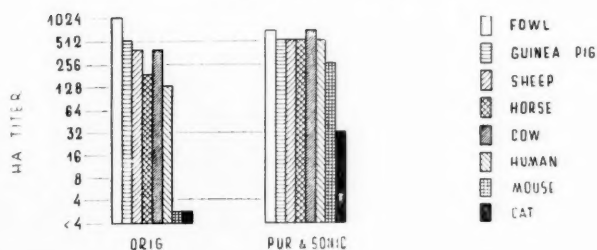
PUR. = partially purified virus preparation

Fig. 2. — Effect of purification of mumps virus (Habel strain) on the capacity of the virus to agglutinate red cells of various species.

very similar manner as described by Cabasso and Cox (3), *i.e.* it did not agglutinate human red cells at any temperature and at +37°C only guinea pig cells were properly agglutinated. According to Cabasso and Cox the Habel strain does not agglutinate chick cells at +37°C. However, in several experiments in our laboratory fowl red cells were «patchily» agglutinated by the Habel strain at +37°C, and this was also the case in the experiment of figure 2. In the purification process part of the hemagglutinins were lost but nevertheless the variations between the titers with different red cells were clearly diminished. The human and mouse titers rose to almost the same level as the fowl and guinea pig titers. However, not even the «purified» Habel virus was capable of agglutinating cat cells.

Experiments like those described above with the Enders and Habel strains were repeated using other virus preparations and red cells. Essentially similar results were obtained.

For the following experiment with the Enders strain the virus preparation was partially purified by low speed—high speed centrifugation as above, but in addition the «purified» viruses were treated in an ultrasonic disintegrator. Red cells from some additional animal species were included. It can be seen (Fig. 3.) that, after treatments of this kind, the Enders strain agglutinates fowl, guinea pig, sheep, horse, cow, and human erythrocytes



ORIG. = original virus fluid
 PUR & SONIC = partially purified virus, subsequently treated with ultra-sonic waves

Fig. 3. — Effect of purification and sonic treatment on the capacity of mumps virus (Enders strain) to agglutinate red cells of various species.

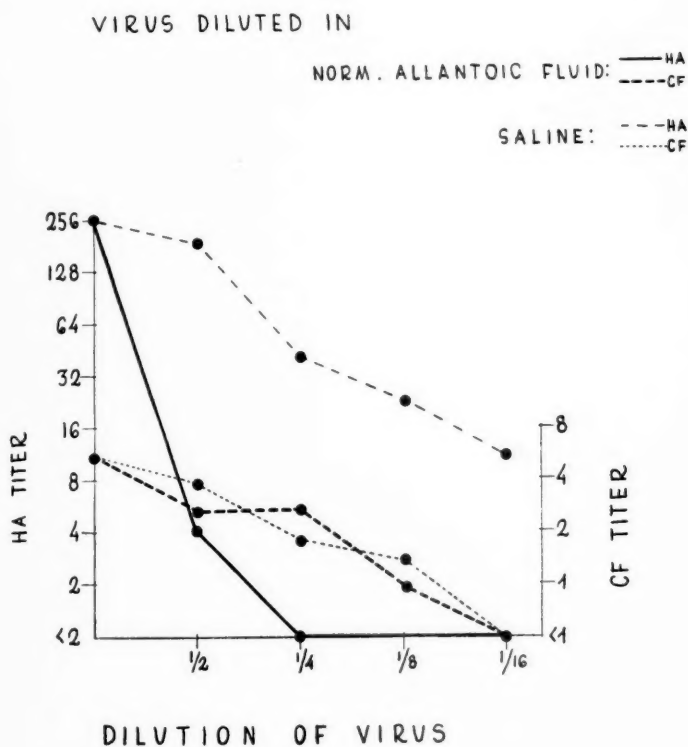


Fig. 4. — Hemagglutinating and complement fixing activities of mumps virus (Habel strain) in the presence of normal allantoic fluid.

equally well. The titers obtained with mouse cells were still somewhat lower and the titers with cat cells definitely lower.

When hemagglutinin (HA) and complement fixation titers (CF) of the same virus allantoic fluids were determined it was found that the HA/CF ratio generally was about 10^1 – 10^2 , *i.e.* not until the hemagglutinin titers exceeded 8–64, antigens could be detected in the CF test. This agrees with the observations with the influenza viruses (13, 14). Paradoxically, however, it was sometimes seen that some nonhemagglutinating infected allantoic fluids could contain demonstrable amounts of antigens in the CF test. To explore the role of hemagglutination inhibitors in these paradoxical observations the following test was carried out: A virus fluid of the Habel strain was parallelly diluted in phosphate buffered saline (pH 7.4) and in normal allantoic fluid (from 14 days' eggs). Both the dilution series were kept for 6 hours at $+4^\circ\text{C}$. The HA and CF titers of the original virus fluid and of all the dilutions were then determined. The results are given in figure 4. When saline was used as diluent the HA and CF titers decreased roughly in the same way. On the contrary, the virus preparation diluted 1 to 4 in normal allantoic fluid was no longer capable of hemagglutinating but still worked as antigen in the CF titration, even in the dilution 1 to 8.

DISCUSSION

It has been suggested that the agglutination spectrum of red cells from various animal species could be utilized in the identification of different strains of Newcastle disease (19) and mumps viruses (3). However, a purification of NDV (7) and mumps virus (results above) from inhibitors can essentially change this agglutination spectrum. The degree of inhibitor contamination, on the other hand, can vary from one virus fluid to another (6). In the authors opinion, these points make the importance of the agglutination spectrum highly questionable as a marker of different mumps virus strains.

The results above indicate that the attachment of the mumps virus to the red cells of different species improves in the following order: cat — mouse — human — fowl. (Cow, horse, sheep and guinea pig are evidently placed between human and fowl in this

cell gradient.) Accordingly, the hemagglutination titers with different red cells are affected by inhibitors in the same order, *i.e.* cat titers decrease first, mouse titers then, and so on. It must be borne in mind that the fowl cells used were taken from «sensitive» hens or roosters. In the case of fowl (16) and sheep (11), but not of man (5), variations have been found in the affinity of red cells from different individuals to mumps virus. Similar inter-individual variations have also been observed in the relation of cow, horse and sheep erythrocytes to NDV (19). Naturally, variations of this kind may cause changes in the order of different species in the cell gradient.

In the earlier paper (6) it was concluded that, the lower the F/H ratio (F = fowl titer, H = human titer) of mumps virus preparation, the smaller is the amount of inhibitor of hemagglutination in the preparation. The results above, however, show that though H and F of the virus fluid of the Enders strain were about equally high (Fig. 1), inhibitor was obviously still present in large amount. The agglutinability of cat red cells was only revealed after a «purification» of the virus preparation; the mouse titers were clearly raised while the human titers remained unchanged. Thus, the F/M (fowl/mouse) and F/C (fowl/cat) ratios obviously reflect the degree of inhibitor contamination of mumps virus preparations more sensitively than does the F/H (fowl/human) ratio. These ratios might well be used as criteria when judging the purity of mumps virus from inhibitors.

The low hemagglutination titers obtained with cat red cells at +4°C and at room temperature and the complete inagglutinability of cat cells at +37°C fully agree with the observations of the agglutinability of cat cells by influenza Lee virus (18). These findings are consistent with the hypothesis of Buzzel and Hanig (2) that cat erythrocytes are characterized by a high negative charge and a low receptor density.

With the exception of cat red cells, the different erythrocytes were agglutinated by the Enders strain equally well at all the temperatures. The Habel strain, on the other hand, at + 37°C agglutinated all the red cells poorly except for guinea pig. This confirms earlier observations (3). The partial purification of the viruses of the two strains further clarified the different hemagglutination behavior of these strains at +37°C. A com-

parison of the enzymatic activities of these strains might throw more light on this dissimilarity. The proper agglutinability of guinea pig cells by the Habel strain at $+37^{\circ}\text{C}$ as compared with the poor agglutinability of fowl cells under the same conditions calls to mind some findings with the influenza virus: the treatment of influenza viruses with ether or with ultrasonic waves can be accompanied by a decrease in hemagglutination titers with chick red cells but by a definite increase in titers with guinea pig cells (14, 15). No valid explanation of these phenomena can be offered, and so the presence of a possible analogy remains open.

When mumps virus was first successfully cultivated in embryonated eggs, the complement fixation test was employed as the criterion of infection (12). The hemagglutinating activity of mumps virus was, however, discovered soon afterwards, and the sensitive and very simple hemagglutination test (HA) almost completely displaced the CF test as an indicator of mumps virus. In this and the earlier paper (6) it has been shown that the great inhibitor sensitivity of the mumps virus restricts the value of the hemagglutination test. Parallel HA and CF titrations (It was pointed out earlier (4) that the antigen measured in the CF titrations probably consisted mainly of V antigen, *i.e.* virus particles.) even showed that situations can occur when mumps viruses are detected by the «insensitive» CF test but not by the «sensitive» HA test.

SUMMARY

The role of inhibitors in the agglutinability of red cells from different species by mumps virus was studied.

Hemagglutination titers of Enders and Habel strains with fowl, guinea pig, human, mouse and cat erythrocytes at different temperatures are presented. Roughly speaking mouse and cat red cells were not agglutinated by the two strains, and human red cells not by the Habel strain. Partial purification of the viruses by low speed—high speed centrifugation clearly equalized the titers obtained with different red cells by raising the lowest titers, *i.e.* those with cat, mouse and human cells.

Partially purified Enders virus subsequently treated with ultrasonic waves gave equally good agglutination of erythrocytes from the following species: fowl, guinea pig, sheep, horse, cow,

and man. The titers obtained with mouse cells remained somewhat lower and titers with cat cells definitely lower even after this procedure.

Virus allantoic fluids made nonhemagglutinating by dilution in normal allantoic fluid could still work as antigens in complement fixation test.

Characteristics of the interaction of mumps virus with red cells of different species and the limitations of the hemagglutination test as a measure of mumps virus are discussed.

It is suggested that ratios between hemagglutination titers with different erythrocytes (*e.g.* fowl/human/mouse/cat) can be used as criteria in judging the degree of inhibitor contamination of mumps virus preparations.

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EFFECT OF THE THYROID HORMONE ON THE UPTAKE OF RADIOSULPHATE (S^{35}) BY THE AORTA IN NORMAL AND CHOLESTEROL FED COCKERELS

PRELIMINARY REPORT

by

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INTRODUCTION

It has been shown in many studies, histochemically and chemically, that changes in the acid mucopolysaccharides of aorta take place in experimental atherosclerosis of animals (7, 10, 12, 25). Mucopolysaccharides are also involved in the formation of atheromas in human atherogenesis (15, 23, 29, 31).

Information on the characteristics of mucopolysaccharides primarily on their metabolism, may be had by means of radio-sulphur. It is known that the deposition of S^{35} in the aorta occurs mainly in the esters of chondroitin sulphates (24). Buck *et al.* (11) found, in 1958, with rabbits, and Kowalewski (20), in 1959, with cockerels, that in cholesterol fed animals the S^{35} uptake by aorta had considerably increased in comparison to controls. Buck (9) demonstrated, by means of autoradiography, that increase in the radioactivity of the aorta results from an abundant uptake of S^{35} by atheromas. It should be noted that in cholesterol fed animals radioactivity has not increased in some other organs into which, nevertheless, plenty of cholesterol infiltrates (11, 20).

The thyroid gland has distinct effects on experimental atherosclerosis (23, 27, 28, 30). It has been demonstrated in several studies that thyroxin reduces and thiouracil increases powerfully the blood

cholesterol. On the other hand, the effect of the thyroid gland on the severity of atherosclerosis itself is less clear and more complicated. It has been proved that thyroxin either reduces, does not affect at all, or even intensifies, the grade of atherosclerosis, depending on the thyroxin dosage (19, 27). As early as 1933 Anitschkow found that big doses of thyroid hormone intensify the severity of atherosclerosis in cholesterol-fed animals (2). Stamler *et al.* (26) advanced the hypothesis that thyroxin has two opposing effects on atherogenesis, viz. that thyroxin inhibits formation of atherosclerosis by decreasing the blood cholesterol, and intensifies it by causing local vascular damage.

The object of the work here reported was to study whether (1) changes in the S^{35} uptake occur even at the initial phase of atherogenesis; in other words, whether, in the uptake of S^{35} in cholesterol-fed animals, changes may be observed in those parts of the aorta where no atherosclerosis is demonstrable macroscopically so far; and (2) whether the thyroid gland has any effect on the uptake of S^{35} by the aorta in normal and cholesterol-fed animals. The object was to study the mechanism of the effect of the thyroid gland on experimental atherosclerosis.

MATERIAL AND METHODS

The experiments were carried out on 5-week-old white Leghorn cockerels which were divided into 6 dietary groups as shown in table 1. Water and commercial mash, which contains 4% of crude fat, the animals had *ad libitum*. The drugs mixed with the feed were methylthiouracil «Orion», 1-thyroxin sodium «Star», and cholesterol «Merck». The birds were killed by decapitation after 6 months of diet.

Determination of S^{35} Uptake. — Radiosulphur (carrier-free sulphate in aqueous solution, diluted 0.5 mC/ml) was injected (0.5 mC/kg of body weight) into the alar vein 48 hours before killing. A sample of about 70 to 80 mg was taken from the aortic arch region at a point where no atheroma could be observed macroscopically. Organic substance was destroyed from the samples by the wet burning method, and S^{35} was precipitated in $BaSO_4$ in accordance with the method presented by Bournsell *et al.* (8). S^{35} is uniformly distributed in the precipitate in this case. The

precipitate was transferred onto an aluminium plate for measuring the radioactivity (precipitate 160 mg/sq.cm). The measuring was performed with an end-Mica-window Geiger-Müller counter. 1600 impulses were counted in each sample; the standard deviation was 2.5 per cent. Radioactivity is expressed in counts per minute/100 mg of wet weight of tissue (cpm/100 mg). Radioactivity of the background, which varied from 12 to 14 counts per minute, has been subtracted from the results.

Autoradiography. — The distribution of S^{35} in the wall of the aorta was examined by contrast autoradiography using the stripping film technique (Kodak Stripping-Plate AR 50). The exposure time was 45 days.

Total Cholesterol of Serum. — Immediately before killing the birds the total cholesterol of serum was determined by means of Abell's method (1).

Grade of Atherosclerosis. — The aortas were classified on the basis of severity of atherosclerosis into groups 1 to 4. In the classification, the most important bases were considered, in accordance with principles advanced by Katz, the size and incidence of atheromas (19). Severity of atherosclerosis in the different groups has been expressed in table 1 by giving the number of the aortas of the total number which exceed grade 2.

The statistical method used is Student's t-test.

RESULTS

The principal results appear in table 1.

In the cholesterol-thiouracil group the weight of the birds began decreasing in comparison to the other groups after 4 months' diet and at the end of the experiment the difference was significant. Between the other groups there was no essential difference in the weight of the birds.

In the cholesterol group the uptake of S^{35} by the aorta even outside atheroma increased significantly ($p < 0.01$). Thus, changes take place in mucopolysaccharides also in what is termed as the initial phase. Thiouracil increases significantly the S^{35} uptake. Thyroxin also tends to intensify it, although the difference is not significant. Both thyroxin and thiouracil intensify the increased uptake of S^{35} by the aorta which is caused by cholesterol.

TABLE 1
THE RESULTS EXPRESSED AS MEANS IN THE DIFFERENT GROUPS AFTER 6 MONTHS' DIET

Dietary Group	Number of Animals	Serum Total Cholesterol, mg %		Severity of Aortic Atherosclerosis > Grade 2/ Number of Animals	S^{35} uptake by Aorta, cpm/100 mg	
		Mean	Standard Deviation of Mean		Mean	Standard Deviation of Mean
I Commercial mash (controls)	19	97	5	1/19	156	8
II Commercial mash + cholesterol 1 %	20	556	88	11/20	188**	8
III Commercial mash + thyroxin 0.00045 %	10	108 ¹		0/10	176	12
IV Commercial mash + thiouracil 0.15 %	8	129	6	0/8	201**	13
V Commercial mash + cholesterol 1 % + thyroxin 0.00045 %	7	395	58	6/7	263***	14
VI Commercial mash + cholesterol 1 % + thiouracil 0.15 %	8	1067	224	5/8	247***	13

¹ pooled

* almost significant $p < 0.05$

** significant $p < 0.01$

*** highly significant $p < 0.001$

Contrast autoradiography showed that the S^{35} uptake by atheroma was abundant (fig. 1). Radioactivity was especially high at margins of atheroma. Even in places where no atheroma but only lipid infiltration existed (in Sudan staining) the amount of S^{35} had increased (fig. 2).

In the thyroxin-cholesterol group the severity of atherosclerosis is slightly more increased than in the thiouracil-cholesterol group, although in the former the total cholesterol of serum is only about one-third of that of the latter group. Small doses of thyroxin would seem to have a tendency to intensify cholesterol-induced atherosclerosis, though the serum cholesterol decreases. It should be noted that the thyroxin dose used in the experiment is small, as no symptoms of hyperthyroidism could be observed in the cockerels.

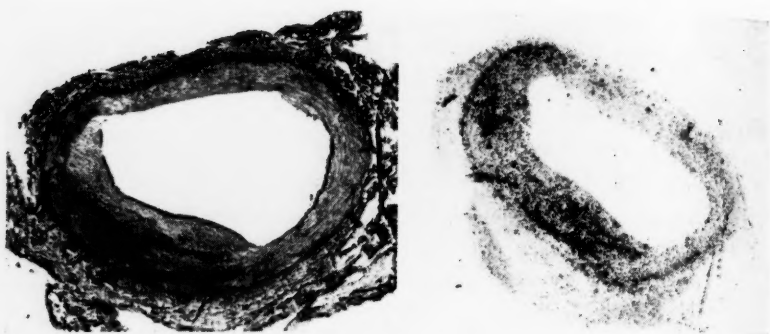


Fig. 1. — On the left: large atheroma in the abdominal aorta. (PAS staining). On the right: autoradiography of the same atheroma. High uptake of S^{35} by the atheroma.

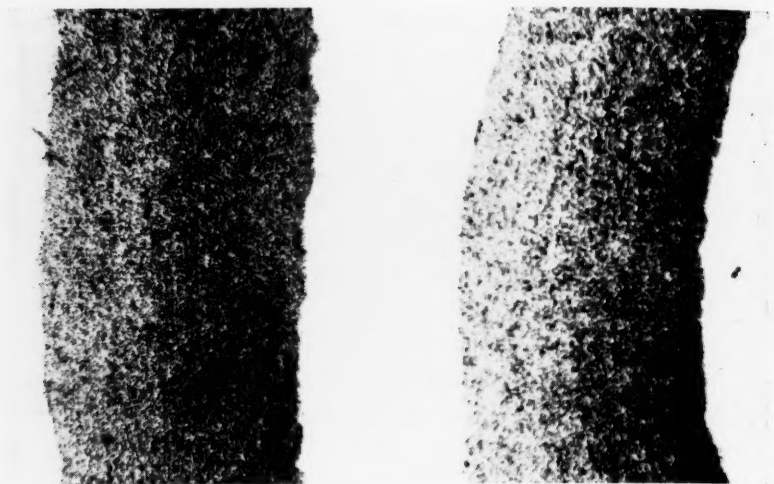


Fig. 2. — On the left: autoradiography of the aorta of a control cockerel. On the right: aorta of a cockerel of the cholesterol-thiouracil group. High uptake of S^{35} in subintimal tissue. In the corresponding place in Sudan staining abundant lipid infiltration.

Thiouracil strongly increases the serum cholesterol, whereas atherosclerosis is increased by it but slightly.

Atherosclerosis cannot be induced by thyroxine and thiouracil alone without a cholesterol diet. On the other hand in the thyroxine group arteriosclerotic changes are observed in the aorta of four cockerels.

DISCUSSION

A number of suggestions have been put forth with regard to the mechanism of lipid infiltration into arterial walls in atherogenesis. Faber (15) thinks that various interfering agents first cause a local increase of mucopolysaccharides in the intima and that lipid infiltration is only secondary. Many authorities believe that a local degenerative lesion in the internal elastic lamina is responsible for the increase of mucopolysaccharides and fibroblasts, which they regard as the «early nonlipid phase» of atherosclerosis (21, 22, 29).

Gore *et al.* (16) and Buck (10) believe that the mucopolysaccharide changes caused by a reaction of the connective tissue of arterial wall due to lipid infiltration are secondary. Gore *et al.* also found, in a series of rabbits, that an increase in metachromasia due to mucopolysaccharides may be found in older atheromas only but none in initial changes which are entirely lipid in nature. Although, on the basis of our results, it is not possible to conclude whether the mucopolysaccharide changes are primary or secondary, we found, however, that changes in the sulphur metabolism of mucopolysaccharides occurred even at an early phase.

The fact that both thyroxine and thiouracil increase the uptake of S^{35} by the aorta need not mean discrepancy, because the S^{35} uptake depends on many different factors, as, *e.g.*, the amount, metabolism and structure (grade of polymerization) of mucopolysaccharides.

Changes in the thyroid activity affect the hypophyseal thyrotropin secretion. It is known that thyrotropin has a direct stimulating effect on connective tissue, which results in an increase of mucopolysaccharides in connective tissue (4, 5). In consequence of continuous hyperthyroidism connective tissue is stained but faintly metachromatically with toluidine blue (3). According to Dziewiatkowski (14), who studied newborn rats which had had thiouracil for 2 or 3 days, the uptake of S^{35} by connective tissue (cartilage) had decreased. Thyroxine, respectively, had an increasing effect or no effect at all, depending on the dosage. The results of this experiment, however, cannot be compared to ours, as the former is concerned with a short-term effect of thyroxine and thiouracil.

Experiments made both with dogs and chickens show that

there is a positive correlation between the degree and duration of hyperlipemia and the severity of atherosclerosis in cholesterol-induced atherosclerosis (6, 18). As appears from our results, this direct correlation between the serum cholesterol and the severity of atherosclerosis disappears when feeding thyroxin and thiouracil.

Thus, it may be assumed that in atherogenesis the thyroid gland has, in addition to its effect on cholesterol metabolism, also another effect, on the arterial wall itself. Holman *et al.* (17) have, in fact, suggested that the arterial wall be considered an active organ whose metabolism and reaction is dependent on many factors, such as hormones and enzymes.

SUMMARY

Cockerels were kept for 6 months on the following diets: (1) cholesterol, (2) thyroxin, (3) thiouracil, (4) cholesterol + thyroxin, (5) cholesterol + thiouracil.

In the cholesterol-fed group the uptake of S^{35} by the chondroitin sulphates of the aorta increased in comparison with the control group. Radioactivity proved to be most powerful in atheromas, but even outside the atheromas the uptake of S^{35} had increased significantly. In other words, changes in the sulphur metabolism of mucopolysaccharides are noticeable even at the initial phase of atherosclerosis.

Thiouracil (prolonged treatment) produces an increased uptake of S^{35} by the aorta. Both thiouracil and thyroxin intensify the increased uptake of S^{35} caused by cholesterol.

Changes in thyroid activity destroy the positive correlation between the serum cholesterol and the severity of atherosclerosis. Thus it may be assumed that in atherogenesis the thyroid gland has, in addition to its effect on cholesterol metabolism, also another direct, effect on the arterial wall itself.

Small thyroxin doses intensify cholesterol-induced atherosclerosis. Apparently thyroxin alters the metabolism and reaction of the aortic wall in such a way that atheromatous formation is facilitated.

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NEVUS OF THE IRIS AS A CAUSE OF ABSOLUTE GLAUCOMA

by

PENTTI MIETTINEN and LAURI MEURMAN

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Nevi are encountered relatively frequently as subsidiary findings in the iris. They do not often attract attention. According to the literature, irinic nevi are not always harmless and may cause various ophthalmic diseases. The majority of the reports concern cases in which nevus has either just become or is just becoming malignant. Coats (2), as early as 1912, published a series of 26 cases in which 7 nevi became malignant during the observation period. Workers have made special efforts to establish whether the formation involved is an actual congenital nevus or one which has developed during life (3 and 4). Mayou (5) reported in 1930 a case in which an irinic nevus was turning malignant. The nevus in this case rose towards the posterior aspect of the cornea and then ran along Schlemm's canal. Duke-Elder (3) reported cases in which the nevus ran along the surface of the iris and spread into the ciliary body and chamber angle. Glaucoma usually ensues in such cases. Duke-Elder emphasised specifically the necessity of following the cases in which glaucoma was associated with nevus of the iris. It must always be considered that the nevus may become malignant. In the ciliary body, nevi usually do not appear clinically but can be stated in connection with histologic studies.

REPORT OF THE CASE

The patient was a farmer's widow of 63 who found the colour of her right eye turning from bluish grey to black. The eye was slightly painful. The pain increased and finally became intolerable. Because of the intense pain the patient had attended the nearest eye hospital in 1954 where the intraocular pressure was found to be 80 mmHg. As conservative treatment failed to lower the pressure, paracentesis of the anterior chamber and iridencleisis were performed. The pressure dropped postoperatively and kept around 50—60 mmHg. As the eye had looked completely black during surgery, the patient was advised to consent to enucleation. The attending physician suspected melanosarcoma. The patient did not, however, consent to enucleation and left the hospital. At the beginning of February 1960, her right again became painful. For this reason the patient came to the Ophthalmic Department, Central Hospital of Kuopio on February 15, 1960. Examination showed vision of her right eye to be 0. Ocular pressure was 80 mmHg. The pupil was deformed and withdrawn upwards (iridencleisis). The limbus showed at 11 and 13 hours 2 post-iridencleisis fragments of the iris. The cornea was opaque throughout. The lens was completely dimmed. The iris looked black through the cornea. The blood vessels of the conjunctiva were markedly dilated.

Vision of the left eye was 1.0. Ocular pressure was 17 mmHg. The pupil was symptom-free. The vitreous was clear and the fundus normal.

Enucleation of the right eye was performed on March 4, 1960, because of intense pain. The entire eye was sent for histologic examination.

Histologic examination (March 14, 1960): Samples prepared from the bulb showed that extremely heavily pigmented nevus cell tissue had infiltrated the connective tissue stroma of the iris in its entirety. From the iris the nevus cell tissue continued to the ciliary body and from there to the chamber angle. In the chamber angle nevus cell tissue was present in dense groups; it thinned into the ciliary muscle and the surrounding connective tissue. In the sclera the collagenous fibres had broken off; they were replaced by loose connective tissue with profuse pigmented nevus cells. Here the nevus cell tissue continued to the subconjunctival connective tissue and under the conjunctival epithelium (previous operations). At this point some of the nevus cells were only slightly pigmented. The fairly dense nevus cell tissue continued from the chamber angle along the posterior surface of the cornea. The cells formed an epithelium-like dense cover which narrowed towards the centre of the cornea. Remains of the lens were visible in the anterior chamber pressed against the posterior aspect of the cornea (Illustration). The dense cell tissue seemed to fill the chamber angle bilaterally. Inflammatory reaction was observed only in the subconjunctival connective tissue. The histologic diagnosis was *nevus pigmentosus*.



Fig. 1. — Nevus cell tissue filling the chamber angle. Remains of the lens visible in the anterior chamber pressed against the posterior surface of the cornea.

DISCUSSION

This was a case in which the colour of the right eye of a farmer's widow of 63 had gradually begun to turn black. The eye became painful and developed absolute glaucoma. Because of elevated pressure, both paracentesis and iridencleisis were performed on the eye in 1954. The ocular pressure remained around 50—60 mmHg for six years and the eye was not painful. In February 1960, the eye again became painful and it was enucleated on March 4, 1960, for pain and suspected melanosarcoma. Histologic examination

showed the causative agent of the condition to be nevus of the iris. The nevus spread into the ciliary body and ciliary muscle and blocked the chamber angle completely. The obstruction of the chamber angle resulted, in turn, in the development of secondary glaucoma. It is impossible to decide whether a congenital nevus was involved, one that had started to grow only when the patient was 57, or whether the nevus had developed later and then begun to grow.

Whichever of the two assumptions is correct, it is certain that the case confirms Duke-Elder's opinion that every irinic nevus must be followed. It is understandable that as long as the nevus remains small it can be regarded as subsidiary ophthalmic finding, but when it begins to grow and especially when the intraocular pressure rises, serious attention must be paid to it.

SUMMARY

A nevus of the iris of a woman of 63 began to grow, blocked the chamber angle and caused absolute glaucoma. The authors emphasise the view that it is necessary to follow every irinic nevus. This is essential especially in the cases in which the intraocular pressure has started to rise.

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INFLUENCE OF ACTH-HORMONE ON THE DEVELOPMENT OF ARTERIOSCLEROSIS

by

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The concept that the adrenal cortical hormones contribute to the degenerative alterations of blood-vessels has a clinical and pathological basis. It has been demonstrated that the hyperactivity of cortex, Cushing's syndrome, is connected with sclerotic alterations of vessels (6). In Cushing's syndrome has been observed the accumulation of cholesterol in the walls of vessels, especially in the intima, and a degeneration of the elastic tissue of the wall (7).

Many series of experiments have been made with ACTH and the hormones of the cortex in experimental research of sclerosis to discover the part the cortex plays in the development of arteriosclerosis. In the experiments, made with these hormones, attention has been paid to their effect on hypercholesterolemia and hyperphospholipemia of the serum. On the changes of the cholesterol and phospholipid contents during the experiment conclusions may be drawn of the possible effect of the hormones on the genesis of sclerosis, though perceptible pathological alterations have not taken place. However, the results of the different experiments on ACTH and the cortical hormones are often dissimilar.

The greatest arteriosclerotic changes with the administration of ACTH have effected Wexler and Miller (12) on rats by injecting

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0.333 IU ACTH per 100 g of body weight three times a week for a period of seven weeks. They also have noted some other changes such as renal calculi, polyarteritis nodosa and gastric ulcers. In an other experiment the same workers had performed unilateral nephrectomy (13) and had thus induced arteriosclerosis in 100 per cent and thrombi in the coronary arteries in 75 per cent with the doses of ACTH mentioned above within a period of 4—7 weeks.

In rabbits ACTH has been found to induce hypercholesterolemia and increasing of lipid fractions of the serum (10). Nevertheless, only one of their forty-eight rabbits had sclerotic alterations. To the results nearly alike came as well Kobernick and More (8) with rabbits as Stamler *et al.* (11) with chicks. On the other hand Pierce and Bloom (9) noted no effect on the cholesterol and phospholipid levels of the serum in rabbits neither Adlersberg *et al.* in dogs (1) by the administration of ACTH.

In human therapy the doses are relatively smaller than in these animal experiments. For instance the dose administrated by Wexler and Miller is ten times bigger than the therapeutic dose. Bloom and Pierce (3) could not observe any alterations in the cholesterol and phospholipid contents of the serum in the patients while using ACTH in therapeutic limits in their experiments. According to Adlersberg, Schaefer and Dritch (2) the cholesterol level of the serum could be reduced by the administration of ACTH for a few days at the beginning of the therapy, but afterwards it increased again.

On account of the fore-going experiments no certain conclusions can be drawn on the effect of ACTH on the development of arteriosclerosis. On the basis of some experiments, however, may be suggested that great doses of ACTH had effect on fat metabolism, because in these experiments the cholesterol and phospholipid levels of the serum had somewhat increased.

The purpose of this experiment was to study with normal rats the effect of a long-time ACTH-treatment on the system of the blood-vessels of the rat, especially on the aorta and the coronary arteries, because in these blood-vessels primarily appears a possible development of arteriosclerosis (4). At the same time attention has been paid to the possible macroscopic changes that Wexler and Miller had observed in their experiments.

MATERIAL AND METHODS

The experimental series consisted of 60 rats, 30 females and 30 males. The females weighed between 185 and 200 g and the males between 250 and 260 g. The animals were approximately eight months old. They were divided into three groups of twenty containing each ten males and ten females.

To the first group was given 0.34 IU per 100 g ACTH (Organon's »Cortrophine» of short duration) dissolved in saline. The amount of the injection was 0.1 ml and the rats were injected subcutaneously in the back.

The second group was injected with saline instead of the hormone. The third group consisted of normal control rats. All the groups were fed on normal diet and given water ad lib.

The duration of the experiment was six months. Six individuals of each group of twenty animals were decapitated after seven weeks and the remaining fourteen animals after six months. As specimens of the blood-vessels the heart with aorta up to the bifurcation was removed. Aorta was separated from the heart in the way that the coronary arteries were left at the side of aorta. The aorta was fixed in 10% neutralized formalin for twenty-four hours and was cut with a freezing microtome in the area of aorta thoracica and at the origin of the coronary arteries. The sections were stained with Sudan III. The kidneys, the heart, liver and intestine were examined macroscopically.

RESULTS

Macroscopic Examination. — During the experiment no animal of any group died. After seven weeks in the heart, aorta and in the blood-vessels of the inner organs a sclerosis could not be found in the macroscopic examination. Neither gastric ulcers in the intestines nor structural and colour changes in the liver could be observed. No renal calculi in the kidneys were encountered.

Even after six months' ACTH-treatment no pathological changes could be observed in the macroscopic examination.

Microscopic Examination. — In the sections made of aorta at the origin of the coronary arteries and in the area of aorta thoracica as well after seven weeks' as after six months' ACTH-treatment

the structure of the intima was coherent and the lumen in cross section was symmetric. In the subintimal tissue, which in the rat is scanty, no proliferation and calcification could be noted. In the media no abnormal structural features could be observed.

DISCUSSION

According to the results of the experiments reported above no changes in the aorta and the coronary arteries indicating sclerosis could be noted in the ACTH treated experimental animals, though it might have been expected on account of the results Miller and Wexler obtained in their experiments on rats (12).

The negative results of our experiment are even more surprising, as our experiment lasted six months, that is three times longer than that of Miller and Wexler. In experiments on rats Gordon (5) has observed after seven weeks' stimulation of the cortex that its reaction capacity in an acid ascorbine test becomes negative. According to him in the blood of the rats some factor is developed, which prevents the reaction of the adrenal cortex. In Adlersberg's *et al.* (2) opinion this substance is possibly an anti-hormone. They consider possible that an ACTH-treatment of a very long duration may produce an antibody reaction, because ACTH is an albumin by nature.

As another possible explanation may be mentioned also the different animals used in the experiments, because the Sprague-Dawley race, used by Miller and Wexler may be more liable to sclerosis than our usual albino rat.

SUMMARY

According to our 6 months' experiment on rats the dose of 0.34 IU ACTH per 100 g, injected three times a week, had no effect on the genesis of arteriosclerosis. No macroscopic alterations could be noted either in the kidneys, intestines or liver.

Acknowledgment. — Thanks are due to Organon for donating »Cortrophine» used in the present study.

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EFFECT OF PSYCHIC STRESS AND CERTAIN HORMONE FACTORS ON THE HEALING OF WOUNDS IN RATS

by

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Stress has a stimulating effect on hormone secretion of the suprarenal cortex (7). Augmented secretion of some corticosteroids, again, has an inhibiting effect on the healing of wounds. The effect of ACTH and cortisone has been studied in especial, and their great inhibitory effect has been noted. Peräsalo & *al.* (5) used rabbits as laboratory animals. Among the rabbits in the group receiving cortisone, wounds healed appreciably slower than among the control animals, whereas in the ACTH group there were no noteworthy changes, the daily dose being 2–6 mg/kg. Alrick & *al.* (1) obtained similar results in experimenting with rats, but ACTH had a retarding effect when the daily dose was 28 mg/kg. On the other hand, 14 mg/kg had no effect. Taylor & *al.* (9) used DCA (desoxycorticosteronacetate), oestradiol and cortisone and observed negative changes only with cortisone. DCA and oestradiol had no effect. Corresponding results in experiments with corticosteroids have been reported by many other researchers (2, 4, 6 and 8). Hormia & Hormia (3) studied the effect of reserpin and chlorpromazin and found that daily reserpin doses of 0.2 and 0.5 mg/kg promoted the healing of wounds to a small extent. In the following we have sought to study mainly the effect of psychic stress, also conjunction with oestradiol, castration, testosterone and reserpin, on the healing of wounds in rats.

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MATERIAL AND METHODS

The material consisted of 124 five-months-old albino rats (Table I).

TABLE I

Group	Number	Sex	Class
1a	9	Male	Control
1b	9	Male	Stress
2a	9	Female	Control
2b	9	Female	Stress
3a	9	Male	Castration
3b	9	Male	Castration and stress
4a	9	Male	Oestradiol
4b	9	Male	Oestradiol and stress
5a	9	Male	Castration and oestradiol
5b	9	Male	Castr., oestr., stress
6a	9	Female	Testosteron
6b	9	Female	Testosteron and stress
7a	8	Male	Reserpin
7b	8	Male	Reserpin and stress

The stress, which was only psychic in nature, was produced by brilliant flashes of light repeated at set intervals and the sharp ringing of a bell as well as a continuous, steady, not very loud noise caused by the scraping of various metal wheels. Each of the b-groups was kept in the stress chamber 2×2 h/day. In other respects, the conditions for by the a- and b-groups were the same.

First the hairs were removed from the hind part of the back, and then a round piece of skin was cut out so as to expose the fascia of the dorsal muscles. The size of the cut was about 900 mm^2 in the case of the males and about 750 mm^2 in the females, which difference roughly corresponds to the difference in the weight between the sexes. The operations were performed after administering ether as an anesthetic and employing an aseptic technique.

The castrations were performed sufficiently in advance for the wounds to heal before the actual experiment was started.

Oestradiolbenzoate was administrated in doses of 0.25 mg a rat per diem.

Testosteronepropionate was administered in doses of five mg a rat daily.

The daily reserpin dose was 0.02 mg.

All three substances were administered intramuscularly and the injections were started seven days before the wounds were inflicted and continued until the end of the experiment.

The healing criterion was a diminishing in the area of the wounds around the edge of the epithelium as well as the date of their complete closing. The outlines of each wound were reproduced on transparent paper, the area being measured on millimeter paper. The measurements were performed at intervals of one week.

At the end of the experiment the rats were weighed and killed. The suprarenal glands were taken out and weighed.

RESULTS

The weights in all the groups increased during the experiment, but no significant differences developed. (Table II).

Likewise, there were no differences between the weights of the suprarenal bodies in groups 1—6; but, compared with the other groups, suprarenal hypertrophy could be noted in the groups receiving reserpin. It may be supposed that the animals have adapted themselves to the stress during the examination because there is no

TABLE II

Group	Body Weight — Gm			Adrenals mg/100 Gm Body Weight	Healing Time Days
	Initial	Final	%Gain		
1a	184	221	20	18.2 ± 0.9 ¹	38.0 ± 1.6 ¹
1b	190	205	17	19.3 ± 1.0	46.2 ± 2.3
2a	162	183	17	20.6 ± 0.7	33.9 ± 2.4
2b	161	179	15	21.8 ± 0.7	35.1 ± 2.5
3a	190	231	22	20.4 ± 0.8	40.1 ± 3.1
3b	193	214	11	20.2 ± 0.7	39.9 ± 1.9
4a	181	218	20	18.9 ± 0.6	40.8 ± 2.0
4b	194	228	18	19.3 ± 0.9	41.1 ± 2.0
5a	187	215	15	19.9 ± 0.9	38.4 ± 2.6
5b	183	212	10	20.2 ± 0.8	38.6 ± 1.4
6a	159	180	13	22.2 ± 0.4	35.7 ± 1.9
6b	164	188	15	22.9 ± 0.7	44.3 ± 4.1
7a	188	212	13	24.0 ± 0.5	35.9 ± 2.2
7b	192	213	11	24.3 ± 0.6	36.6 ± 3.0

¹ Standard deviation

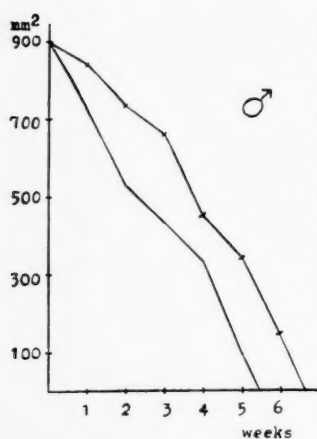


Fig. 1.

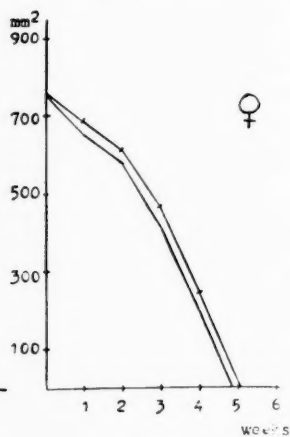


Fig. 2.

- group 1a, male control rats
 —|—————| group 1b, male stress rats
 ————— group 2a, female control rats
 —|—————| group 2b, female stress rats

adrenal hypertrophy in the stress groups compared with the control groups. (Table II).

1a and b. Among the control males healing lasted 38.0 ± 1.6 days. Among the stress males the period was 46.2 ± 2.3 days. Thus, marked retardation could be noted. (Fig. 1).

2a and b. The healing times in the female control and stress groups were 33.9 ± 2.4 and 35.1 ± 2.5 days respectively — in other words, no real difference could be said to have been evident. (Fig. 2).

3a and b. In the group consisting of castrated rats, the healing time of the animals exposed to stress was roughly the same as in the case of the corresponding a-groups, the periods being 40.1 ± 3.1 and 39.9 ± 1.9 days. Moreover, comparison with the control group 1a revealed no significant difference. (Fig. 3).

4a and b. The time it took the oestradiol group to heal was 40.8 ± 2.0 days, the period of the oestradiol group exposed to stress being 41.1 ± 2.0 days. There is thus no appreciable difference, nor is there any in a comparison with the control group. (Fig. 4).

5 a and b. The group which was castrated and received oestradiol required 38.4 ± 2.6 days for its wounds to heal and close, the period for the corresponding stress group being 38.6 ± 1.4 days. The difference is insignificant, also in respect to the controls. (Fig. 5).

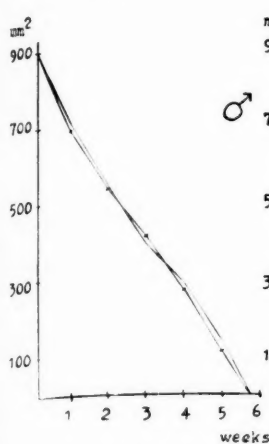


Fig. 3.

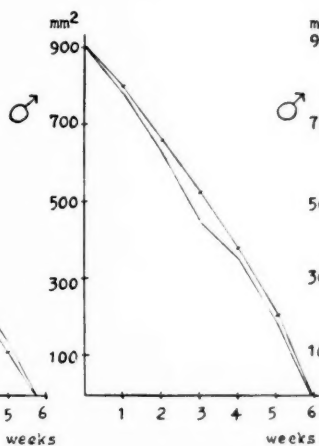


Fig. 4.

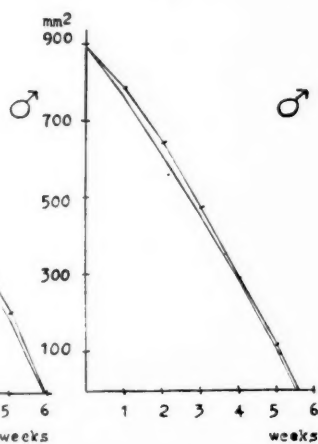


Fig. 5.

- group 3a, male rats, castrated
- - - group 3b, male rats, castrated and stress-exposed
- group 4a, male rats, oestradiol-treated
- - - group 4b, male rats, oestradiol-treated and stress-exposed
- group 5a, male rats, castrated and oestradiol-treated
- - - group 5b, male rats, castrated and oestradiol-treated and stress-exposed

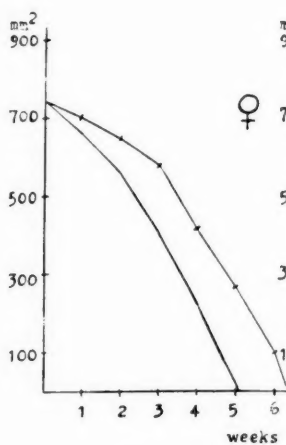


Fig. 6.

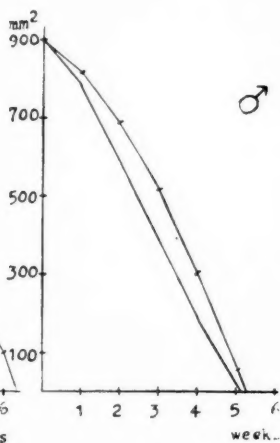


Fig. 7.

- group 6a, female rats, testosterone-treated
- - - group 6b, female rats, testosterone-treated and stress-exposed
- group 7a, male rats, reserpine-treated
- - - group 7b, male rats, reserpine-treated and stress-exposed

6a and b. These groups, which consist of females, received testosterone. The healing time of the a-group was 35.7 ± 1.9 days, whereas in the case of the b-group it was 44.3 ± 4.1 days, signifying that stress had a marked retarding effect. The result in the a-group did not differ from that of the controls. (Fig. 6).

7a and b. Male rats receiving reserpin. The healing time of the a-group was 35.9 ± 2.2 days, which does not deviate from the controls. But the period of 36.6 ± 3.0 days for the b-group is distinctly shorter than the time it took for the control rats exposed to stress to heal. (Fig. 7).

CONCLUSIONS

1) The wounds of female rats heal under normal circumstances slightly faster than those of males. Although the wounds inflicted on the females differed in size from those of the males, the results can perhaps be regarded as comparable inasmuch as this difference corresponds to the animals' difference in weight. Among the female animals the healing time was 33.9 ± 2.4 days and among the males 38.0 ± 1.6 . The average difference is thus 4.1 days.

2) Psychic stress retards the healing of wounds in male but not female rats. Among the males the average difference between the stress-exposed and control rats is about 8 days.

3) Oestradiol and castration, each separately, eliminate the retarding effect of stress in male rats but do not promote healing when administered to rats existing under normal conditions. This agrees with the observation made by Taylor & *al.* concerning the effect of oestradiol. No differences between the effects of castration and of oestradiol could be observed in our tests, nor were any apparent in comparison with the group which had been castrated and received oestradiol either.

4) Testosterone as administered to female rats under normal conditions does not retard the healing of wounds, but it does under conditions of stress, when an average retardation of about 9 days occurs.

5) Reserpin does not affect the healing of wounds under normal conditions, but under conditions of stress its effect is to promote healing. A difference of about 10 days was obtained in the experi-

ment upon comparing reserpin and normal male rats under stress conditions. Our results are not in conflict with the observations of Hormia & Hormia, for in their investigation too the effect of reserpin in promoting healing under normal conditions was minimal.

It may thus be noted that among female rats receiving testosterone the healing of wounds proceeds in the same way as among males under corresponding circumstances, and among oestradiol-treated and castrated male rats in the same way as among females. Testosterone in a sense sensitizes rats to the effects of psychic stress as far as the healing of wounds is concerned. Oestradiol again eliminates this sensitization.

SUMMARY

In the present investigation, which was carried out with 124 rats, it was observed that skin wounds normally heal slightly faster in female than male animals. Psychic stress retards the healing process in male but not female rats. Oestradiol and castration eliminate the retarding effect of stress in males. Testosterone again retards the healing of wounds in females under conditions of stress. Reserpin has the effect of hastening the healing process in rats exposed to stress, whereas under normal circumstances it has no effect.

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EFFECT OF INFLUENZA ANTISERA ON THE ENZYMATIC ACTION OF FIVE INFLUENZA A VIRUSES¹

by

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The present writer has shown previously that influenza A/F/51 and Lee antisera, even in very weak dilutions, inhibit the enzymatic effect of homologous virus on egg white inhibitor. They had no effect, however, on the enzymatic action of heterologous virus (1).

The object of the present work was to ascertain whether antisera inhibit the enzymatic action of influenza viruses in the same way as they inhibit the hemagglutination caused by them. Were the inhibition of enzymatic action different from that of hemagglutination, it might imply that the mechanism of action of antiserum against these different actions of the influenza viruses is not, at least not completely, similar and that »enzyme inhibition» can be used to throw additional light on the antigenic properties of influenza viruses. Five influenza A viruses and their homologous antisera and 2 other influenza antisera were studied.

MATERIAL

Virus Strains. — The influenza virus strains were PR₈, FM₁, A/Finland/ 51/1 (A/F/51), A/Finland/53 (A/F/53) and A₂/Finland/57 (A₂/F/57). All were egg strains.

¹ Aided by a grant from the Sigrid Jusélius Foundation.

Antisera. — The antisera were prepared by immunising chicken with the above strains and also with influenza virus strains A/Finland/57/15 (A/F/57) and B/Finland/55 (B/F/55). The hemagglutination inhibition titres ranged from 400 to 6400. Before the test, the sera were treated with Cholera Vibrio enzyme (Philips Roxane, one-sixth of serum) for 20 hours at +37°C, after which the enzyme was destroyed by keeping the sera for 1 hour at +56°C.

Egg Inhibitor. — This was prepared from filtered egg white by precipitating it with distilled water (1.5 volumes) at +4°C. The precipitate was washed twice with distilled water and finally made up to the initial volume in saline and centrifuged clear (1500 RPM, 5 min.). The inhibitor titre range was 1200—2400.

Indicator Virus. — Lee strain of B influenza virus heated for 30 min. at +56°C.

Reaction Medium. — Laboratory broth of the same composition as in the earlier investigation (1).

Inhibition Titration Medium. — Saline buffered with 0.06 M phosphate buffer (1/10 part buffer).

Red Cells. — 0.25% chicken red cell suspension.

METHODS

Titration of Inhibitory Activity. — The titre of egg white inhibitor was determined by titrating with indicator virus on a plastic plate, as described above (1). The inactivation of the inhibitor in the tests was determined by examining the inhibition titre before and after the test.

Hemagglutination Inhibition Cross Test. — The tests were performed on a plastic plate, making a two-fold dilution series of the antisera on the plate in 0.25 per cent red cell suspension to a volume of 0.25 ml and adding to this 4 agglutinating doses of the virus under examination in a volume of 0.25 ml. The tests were carried out at room temperature. The result was read after one hour. Each virus was studied with each antisera.

Enzyme Inhibition Cross Test. — The enzymatic activity of the viruses was first studied in the earlier manner (1). The strength of the different viruses selected was that which caused complete inactivation of the inhibitor. It varied with the viruses in the

dilution range 10^{-3} — 10^{-4} . The final dilutions of the antisera were 1/60, 1/600, 1/6000 and 1/60000. Every virus was tested against every antiserum. The tests were performed in the same manner as in the investigation mentioned previously (1).

RESULTS

The results are given in Tables 1 and 2.

TABLE 1
HEMAGGLUTINATION INHIBITION CROSS TEST. THE FIGURES DENOTE THE MAXIMUM FINAL ANTISERUM DILUTION WHICH INHIBITED HEMAGGLUTINATION

Antiserum	Virus				
	PR ₈	FM ₁	A/F/51	A/F/53	A ₂ /F/57
PR ₈	6400	<24	<24	24	<24
FM ₁	200	1600	100	<24	<24
A/F/51	<24	1600	3200	800	<24
A/F/53	<24	<24	24	400	<24
A/F/57	<24	<24	<24	200	<24
A ₂ /F/57	<24	<24	<24	<24	3200
B/F/55	<24	<24	<24	<24	<24

The results obtained in the hemagglutination inhibition tests indicate the gradual changing of the antigenic properties of influenza A viruses until the Asia strain is clearly distinguished from the other strains.

Using the enzyme inhibition test it was possible to demonstrate the effects of very small quantities of antiserum, even 1/60000 homologous antiserum. On the other hand, 1000 times greater amounts of antiserum had no effect on the enzymatic action of heterologous viruses.

The results obtained in both the tests were very similar. The antisera inhibited parallelly both the hemagglutination and enzymatic action of the viruses. Distinct differences, too, were observable in places. FM₁ antiserum had a fairly strong inhibitory effect on the hemagglutination of PR₃ virus but not on its enzymatic action. A/F/53 antiserum, again, did not inhibit hemagglutination

TABLE 2

ENZYME INHIBITION CROSS TEST. THE FIGURES DENOTE THE PERCENTUAL QUANTITY OF INHIBITOR NOT INACTIVATED

Antiserum Dilution	Virus					
	PR ₈	FM ₁	A/F/51	A/F/53	A ₂ /F/57	
PR ₈	60	100	<1.5	12	50	<3
	600	100	<1.5	6	25	<3
	6000	100	<1.5	3	25	<3
	60000	25	<1.5	3	12	<3
FM ₁	60	3	100	50	50	<3
	600	<1.5	100	50	25	<3
	6000	<1.5	100	25	25	<3
	60000	<1.5	<1.5	12	12	<3
A/F/51	60	3	100	100	100	<3
	600	<1.5	100	100	100	<3
	6000	<1.5	6	100	100	<3
	60000	<1.5	<1.5	100	50	<3
A/F/53	60	<1.5	100	50	100	<3
	600	<1.5	100	12	100	<3
	6000	<1.5	<1.5	12	50	<3
	60000	<1.5	<1.5	6	25	<3
A/F/57	60	1.5	50	50	100	<3
	600	<1.5	<1.5	25	100	<3
	6000	<1.5	<1.5	6	50	<5
	60000	<1.5	<1.5	3	25	<3
A ₂ /F/57	60	1.5	<1.5	3	25	100
	600	<1.5	<1.5	3	25	100
	6000	<1.5	<1.5	3	25	50
	60000	<1.5	<1.5	3	25	25
B/F/55	60	<1.5	<1.5	12	25	<3
	600	<1.5	<1.5	6	25	<3
	6000	<1.5	<1.5	6	25	<3
	60000	<1.5	<1.5	3	25	<3

FM₁ virus but it inhibited enzymatic action nearly to the same extent as it inhibited the action of homologous virus. B/F/55 antiserum inhibited the enzymatic action of A/F/51 virus to some extent. Furthermore, a few minor differences were observed. A₂/F/57 antiserum in both the tests had an inhibitory effect only on the homologous virus. Analogously, only homologous antiserum affected the Asia virus.

DISCUSSION

The ability of antiserum to inhibit the enzymatic action of a virus but not its hemagglutination might be due simply to the longer reaction time or to the fact that the quantity of antiserum used in the enzyme inhibition test was large compared with the viral amount. The maximum quantity of antiserum employed in the enzyme inhibition test (1/60 dilution) was considerably greater in ratio to the virus used than the maximum quantity of antiserum (1/24 dilution) used in the hemagglutination inhibition test. There is evidence, however, that the explanation is not simply the difference of a long reaction time or a relatively greater quantity of antiserum. This is seen from the fact that the results in the two tests were not always the same. Antiserum FM₁, for instance, inhibited the hemagglutination of PR₈ virus but not its enzymatic action. Antiserum A/F/53 was a considerably stronger inhibitor of the enzymatic action of FM₁ virus than of A/F/51 virus, whereas in the hemagglutination inhibition test the same antiserum inhibited A/F/51 virus slightly but FM₁ virus not at all.

It would seem that the enzyme inhibition test can be used to study the antigenic properties of influenza viruses and that it can elicit properties of the viruses that are not brought out by the routine hemagglutination inhibition test. The method is somewhat complicated, but hardly any more troublesome than some other tests employed to the same end.

SUMMARY

The effect of some influenza antisera on the enzymatic activity of 5 influenza A viruses was studied. The results were compared with the results of the hemagglutination inhibition test performed on the same antisera and viruses. The effect of the antisera was similar in both the tests. There were also, however, differences distinct enough to warrant the assumption that the mechanism of action of the antiserum is not fully identical in the two tests. The enzyme inhibition test may be suitable for study of the antigenic properties of influenza viruses.

LITERATURE

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EFFECT OF SAUNA BATH ON QUICK'S PROTHROMBIN TIME

by

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According to the study of Lyburne a hot environment causes a prolongation of the prothrombin time of Quick up to many times normal (1). In his experiments the persons tested were shut in a hot-air box (2) for about 40 minutes in a mean temperature of about 56° (from 71° falling to 47°) C. When the 5 people tested were given an anticoagulant drug (Marcoumar) before the experiment, the prolongation of the prothrombin times was further multiplied by 1½—5 times.

As sauna bathing is a common habit of people in this country the observations of Lyburne may be of some practical significance here. It is quite usual for patients who have recovered from an acute episode of thrombosis or embolism and are still going on with an anticoagulative therapy, that they return, as far as possible, to their former habits of, among other things, sauna bathing. In fact, one of the most common questions put to the physician by a patient who has recovered from an acute myocardial infarction is: — may I take sauna baths?

The tolerance of patients who have recovered from myocardial infarction to the Finnish sauna has been studied by Räsänen (3). The aim of the present study was to shed light on the question, has sauna bathing any effect on the prothrombin time, and, if so may it be harmful or even dangerous to patients treated with anticoagulants?

The study has been supported by Yrjö Jahnsson's Foundation.

METHOD OF STUDY

The determinations of prothrombin times were made by the Quick-Lehmann method as double determinations, each of which included 3 readings of the coagulation time. The standard deviation of the double determinations was counted to be $S. D. = \pm 0.90$ sec., and from this the standard error of the method, $S. E. = \pm 0.182$ sec.

In the first experiment, 7 healthy men, (one of which had, however, a considerable emphysema caused by kyphoscoliosis), were exposed to heat in an ordinary Finnish sauna bathhouse sitting on the platform for 15 minutes in a heat of 85°C , the relative humidity as measured by a hair-hygrometer decreasing from the initial 80% to 65%. The corrected atmospheric pressure was 758 mm Hg. The temperature of the sampling room was 23°C , that of the cooling water (in a lake) 12° and that of the air outdoors 14°C .

The samples for the prothrombin determinations were taken from the antecubital veins before the exposure to heat, immediately after the stay on the sauna platform, and again after 15 minutes of cooling.

The second experiment was performed under similar conditions except that the prothrombin times of the 5 men under test were prolonged by the administration of a 2 weeks course of an anticoagulant (Marcoumar, Hoffman-LaRoche) as follows: 15 mg of the drug were given the first day, 9 mg the 2nd day and later on 3—6 mg a day, to reach and maintain a steady and reasonable prothrombin time level of 2 to 3 times that of normal (22 sec.). The temperature in the sauna this time was 60°C , the relative humidity 60%, and exposure time 40 minutes including 3 cooling pauses of two minutes duration. The blood samples were taken before entering and immediately after leaving the hot room but no observations were made this time of the effect of cooling.

In the third test the same 5 men stayed in the sauna for 45 minutes while the temperature in the room gradually decreased from 89° to 72°C , averaging about 80° , the relative humidity was 30% and the atmospheric pressure 756 mm Hg. Wiping of the heads with wet snow was allowed for cooling because such heat could otherwise not have been tolerated continuously. 4 persons tested out of 5 reported they felt ill and weak after the experiment. The loss of weight of these 5 persons was in all 6 kg, thus averaging 1.2 kg per person. Prothrombin samples were taken (without any previous therapy with anticoagulants) before and immediately after the stay in the heat.

In this way it was aimed to investigate the effect of different methods in sauna bathing: 1) a short period in tense damp heat, 2) moderate damp heat of long duration, 3) in tense dry heat of long duration.

RESULTS

The results of the first sauna test are shown in table 1. The prothrombin times of all 7 people tested decreased slightly, an average -2.6 seconds ($p < 0.02$).

TABLE I

Object	Prothrombin time in Seconds		
	Before	After Sauna Bath 15 Min. 85°C	
		Immediately	Cooling 15 min.
V. B.	24	23	23.5
P. R.	23	19	22
J. J.	23	22	23
K. H.	24	22	23
K. O.	22	19	21
A. H.	23	20	20.5
E. W.	26	22	22
Average change		-2.6	$+1.1$
S. D.		± 1.27	± 0.76

The effect of sauna bathing on the prothrombin times, previously prolonged by treatment with Marcoumar, is illustrated in figure 1. In 4 cases a slight increase in the prothrombin time was noted but in one a decrease of 10 seconds was observed. The average change was $+2.2$ seconds, which is not a significant difference.

The results of the third sauna test is shown in table 2, from

TABLE II

Object	Prothrombin Time in Seconds	
	Before	After
E. W.	24	21
V. B.	21	20
A. H.	22	18
K. H.	21	20
E. S.	25	23
Average change	-2.2	
S. D.	± 1.24	

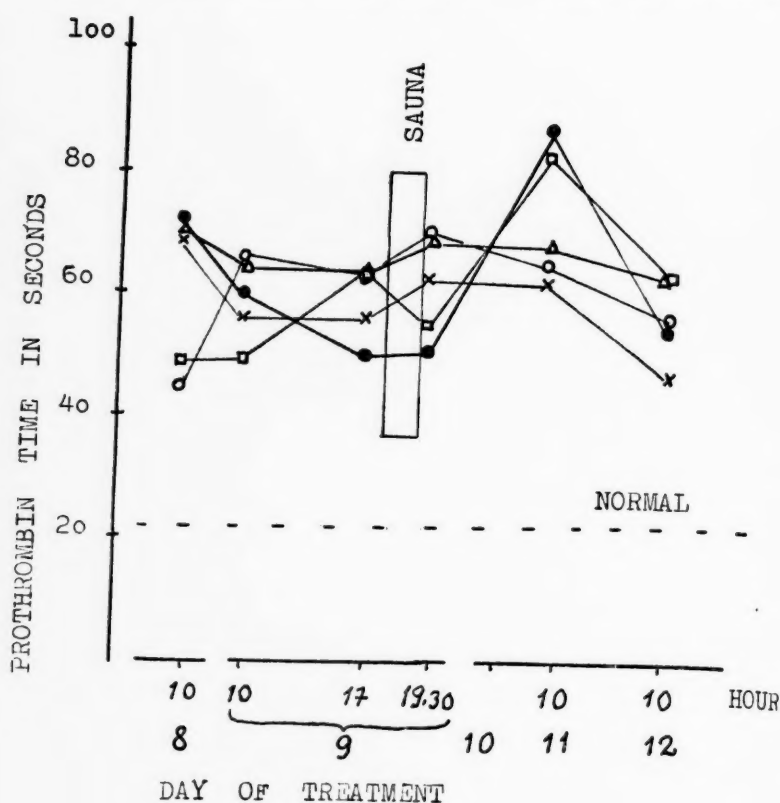


Fig. 1.

which it is seen that the coagulation times of all 5 men decreased a little, on average — 2.2 seconds, an almost significant difference ($p < 0.05$).

DISCUSSION

As far as known to the authors, no experimental work has been recorded on the effect of sauna bathing on the coagulation mechanism. Tawfic (4) has observed an increased bleeding tendency in tropical climates in persons treated with anticoagulative therapy (phenylindandion). Changes have also been observed in the fibrinolytic activity^{*} of the blood effected by exposure to heat in the sauna (7). Lyburne (1) used a hot-air box for heating as

described by him in an earlier publication (2). Marcoumar was used as anticoagulant, and a modification of the Quick method was used for the determination of prothrombin. It seems to be very probable that, from physiological point of view, the effect of Lyburne's heat box and the Finnish sauna ought to be the same. Lyburne reported, however, that considerable prolongations of prothrombin times were observed, whereas in the present study the prothrombin time was slightly shortened. Our experiments were performed on healthy men 26 to 44 years of age while Lyburne's experiments were carried out on patients aged 56 to 70, who were suffering from heart and/or circulatory diseases. One cannot, however, understand the prolongation of the prothrombin time of Quick (properly thromboplastin time) caused by heat in these patients. Strong neurovegetative impulses, like shock and collapse are able to influence changes in the coagulation mechanism and cause prolongation of the coagulation time (5), and metorotrophic irritants may cause biphasic changes in coagulation activity in animal experiments (6). But it does not seem likely that the above mentioned diseases could cause a considerable prolongation of the coagulation time, even through a hypersensitive neurovegetative reflectory system, as there were no signs of such phenomena in the healthy persons who were exposed to heat in a sauna to the point of collapse.

Quick's method measures mainly the activity of the factors V and VII and to a much lesser degree that of prothrombin, fibrinogen and factors VIII, IX, X, XI and the PTA factor (5). The so called prothrombin time is dependent on the concentrations of prothrombin, factors V, VII, IX, fibrinogen and antithrombin, and it would be more correct to call it «thromboplastin time» (5). Thus it may be the liability to error of Quick's method of determination of the prothrombin time that has led to conflicting results. Lyburne's tables show that anticoagulative drug therapy did not increase the anticoagulant dispositions caused by heat. On the contrary, in the patients, who were pretreated with Marcoumar, heat prolonged the time *less* than in persons with no previous therapy.

The probable explanation of the slight decrease in the prothrombin time caused by heat in the sauna, as observed in the present work, seems to be simply dehydration.

On the basis of our study we conclude that the Finnish sauna bath ought not to cause an increased risk of bleeding to a person treated with anticoagulants.

Further studies will show, if the above conclusion holds, when more accurate and specified methods are used, and patients with thromboembolic diseases are investigated.

SUMMARY

The effect of the heat of the Finnish Sauna bath on the prothrombin time of Quick (thromboplastin time) in healthy men has been studied.

A slight but significant decrease in the prothrombin times was observed. When previously treated with an anticoagulant drug, no significant change was noted in the prothrombin times due to the Sauna bath.

We conclude that the Sauna bath does not cause, so far as is known, an increased risk of bleeding to people treated with anticoagulative therapy.

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PRESENCE OF A PULMONARY FUNGUS IN RODENTS IN FINLAND

by

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The presence of a pulmonary fungus, *Haplosporangium*, in rodents in Sweden was reported by one of us (W. L. J.) in 1956 (3). The name of this organism has since been changed to *Emmonsia* with the description of a new genus by Ciferri and Montemartini (1). However, it seems desirable to retain the term haplomycosis for the disease associated with this fungus. The specimens from Sweden came from one wood mouse, *Apodemus flavicollis*, collected in Gävleborg County. In 1959 additional infected animals were found in Sweden, cultures were established and the identification confirmed. Infected animals were also found in Norway (manuscripts in press).

The search for this pathogen was also extended to Finland in April and May 1959 and the laboratories at the Finnish Game Research Institute, Helsinki, were utilized. Some technical assistance was received from the State Veterinary Institute at Helsinki.

Trappers in southern Finland were asked to send in fresh carcasses of muskrats, *Ondatra zibethica*, after they had removed the pelts. A zoology student, Mr. Uolevi Skaren, was employed to trap water rats, *Arvicola terrestris*, and other small mammals in the vicinity of Helsinki. A considerable number of animals preserved in

TABLE 1
ANIMALS EXAMINED FOR HAPLOMYCOSIS IN FINLAND, MAY 1959

	Fresh Specimens	Museum Specimens	Total	Total Infected
Voles, <i>Microtus</i> spp.	1	59	60	1 (fresh)
Red-backed mice, <i>Clethrionomys</i> sp.	0	27	27	0
Muskrats, <i>Ondatra zibethica</i>	35	0	35	2
Water rats, <i>Arvicola terrestris</i> ..	12	7	19	7 (5 fresh 2 museum)
Lemming, <i>Lemmus lemmus</i>	0	10	10	0
Hare, <i>Lepus</i> sp.	1	0	1	0
Total	49	103	152	10

formalin or alcohol at the Zoology Museum of the University, Helsinki, were also examined with the permission of the curator, Dr. S. Segerstråle. From April 29 to May 7, 152 small mammals were examined as listed in table 1. All of the species examined were animals indigenous to Finland except the American muskrat, *Ondatra zibethica*, which was introduced into Europe and Asia and which is now widespread.

Of the 152 animals autopsied, 10 were found infected with *Emmonsia*¹. Both preserved museum specimens and freshly trapped animals were found infected. Collection data for the infected animals are as follows:

No. 360. — Mature muskrat ♀, *O. zibethica*, Siilinjärvi Commune, Finland. May 4, 1959. Two spherules in portion of lung examined measured 170 μ and 244 μ in diameter.

No. 363. — Mature muskrat, *O. zibethica*, Commune of Koirjärvi, Finland, April 30, 1959. Very heavy infection throughout the lungs (figure 1). Cultures established.

No. 366 A and B. — Water rats, *A. terrestris*, Korsholm Island, in Sipoo about 25 miles east of Helsinki, Finland, August 27—28 1954, collected by the late Prof. I. Välikangas. Five rodent specimens preserved in alcohol at the Zoology Museum, Helsinki. Two of these five were infected, one with very numerous spherules, one with a few scattered spherules.

No. 369. — Water rat ♂, *A. terrestris*, near Helsinki, Finland, May 5, 1959. Moderate infection. Ten spherules dissected out and measured were 429 μ maximum, 384 μ minimum, and 414 μ average diameter.

¹ Use of the specific name for this parasite has been avoided because of certain taxonomic revisions now in the process of publication.



Fig. 1. — No. 363, *Ondatra zibethica*, Koijärvi, Finland, April 30, 1959. Gross specimen of lungs with heavy infection of *Emmonsia*. About 2 X magnification. Photo by Photographic Laboratory, University of Helsinki.



Fig. 2. — No. 363. *Ondatra zibethica*, Koijärvi, Finland, April 30, 1959. Histological section of lung. About 18 X magnification. Many spherules are present, some are crushed by microtome. Photo by N. J. Kramis.

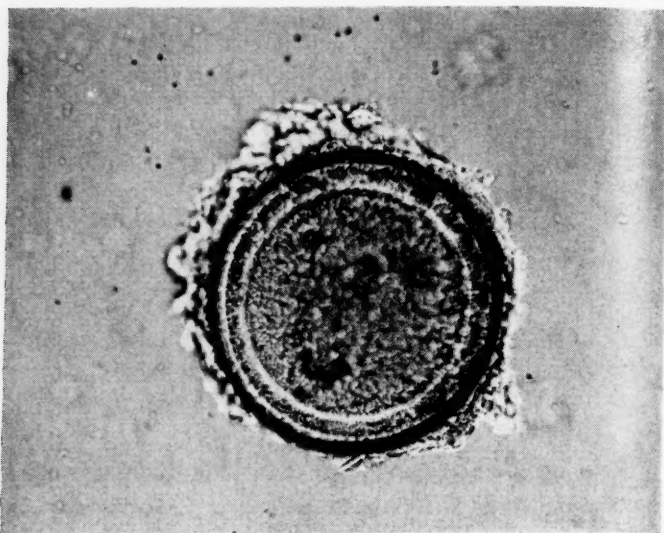


Fig. 3. — No. 363. *Ondatra zibethica*, Koijärvi, Finland, April 30, 1959. Optical section of spherule in lung. Magnification about 136 X. Photo by N. J. Kramis.

No. 371. — Vole, large ♂, *Microtus agrestis*, near Helsinki, Finland, April 5, 1959. Two spherules only, 326 μ and 348 μ diameter.

No. 372. — Water rat, *A. terrestris*, near Helsinki, Finland, May 6, 1959. Heavily infected. Portion of lung sent to Dr. Anderson, D. V. M., State Veterinary Institute, Helsinki, for culture and pathologic study.

No. 373. — Water rat, *A. terrestris*, near Helsinki, Finland, May 6, 1959. Light infection with spherules. Minimum 296 μ , maximum 407 μ , average 353 μ , diameter.

No. 375. — Water rat, *A. terrestris*, near Helsinki, Finland, May 7, 1959. Infected.

No. 377. — Water rat, *A. terrestris*, near Helsinki, Finland, May 7, 1959. Infected.

Sixty-nine of the specimens of *Microtus*, *Clethrionomys*, and *Lemmus*, which were preserved at the Zoology Museum, came from Kilpisjärvi in the extreme northwestern part of Finland. None of these was found infected.

DISCUSSION

In 1942, an epidemic of nephritic disease occurred among troops in Finnish Lapland. Both Finnish and German troops were involved

as reported by Hortling (2) and Stuhlfauth (6). The etiology in this epidemic was never established and although the disease was thought to be a form of leptospirosis, this could not be verified. There is some question as to whether this is the same disease as has been described independently by Myhrman (5) in Sweden and Muri (4) in Norway and referred to as «nephropathia epidemica». The etiology of nephropathia epidemica is likewise obscure.

Both Hortling and Stuhlfauth thought that the human illness in Lapland was associated with the numerous wild mice and lemming which were present that year. Infection in Norway and Sweden also was attributed to wild rodents. One of the authors (W.L.J.) developed the hypothesis that there may be an association between these human diseases of unknown etiology and prior exposure of the patients by inhalation of spores of *Emmonsia*.

The presence of this disease or these diseases in Finland and the other Scandinavian countries prompted the present study of wild rodents. As several factors in the epidemiology of the disease parallel those of known mycotic infections, special attention was given to searching for fungus agents in the rodents. As a result of these studies, haplomycosis, caused by the fungus *Emmonsia*, was found to be widespread and relatively abundant in three genera, *Arvicola*, *Microtus*, and *Ondatra*, of microtine rodents in southern Finland.

At this writing (September 1959) human infection with *Emmonsia* has not been recognized.

SUMMARY

The pulmonary fungus *Emmonsia* was found in 10 animals representing 3 genera of rodents in Finland in 1959. The hosts were *Arvicola terrestris*, *Microtus agrestis*, and *Ondatra zibethica*. Cultures of the fungus were established and identification confirmed by study of the mycelial phase.

The search for rodent pathogens in Finland and for *Emmonsia* in particular was undertaken because of the presence of a «field fever» of man of unknown etiology. This disease is attributed to association with rodents. A similar or identical disease «nephropathia epidemica» in Norway and Sweden is likewise attributed to association with rodents, but the etiology and mode of infection are entirely unknown. There are some characteristics in the epidemiol-

ogy of these diseases that suggest a fungus etiology. The possibility of an association between this agent and these human diseases is suggested.

At this writing (September 1959) *Emmonsia* has not been recognized as a human pathogen.

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EFFECT OF A MECHANICAL ABDOMINAL STRESS ON ADRENAL CORTEX

A COMPARISON BETWEEN THE EFFECTS OF LIGATION OF THE UPPER
AND LOWER PART OF THE GASTROINTESTINAL TRACT

by

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Many gastrointestinal diseases, such as peptic ulcers, are one form of manifestation of the general adaptation syndrome (10, 11, 12). On the other hand, mechanical gastrointestinal disturbances can act as a non-specific stress to the organism. The pathophysiological consequence of an acute obstruction of the small intestine is the ischemia caused by the increased intraluminal pressure of the intestine, edema and increased capillary permeability. The absorption of gas and fluid is disturbed in the process, which finally leads to dehydration and azotaemia. As to the chemical imbalance, the process takes place more slowly in the passage disturbances of the large bowel; at a later stage, however, a disturbance is coming with from the small intestine (4, 18). An inflammatory component due to bacterial invasion to the intestinal mucosa is also influencing in obstructive processes (*e.g.* 9, 18). The peritoneal pain is coming with as a component in the abdominal stress formed in this way. The significance of pain as a non-specific stress is commonly known (10, 12), and functional disturbances in the endocrine glands and activation of the suprarenals have been observed in experimental animals (13).

The alarm reaction as a consequence of a non-specific stress manifests itself in the histological features of adrenal cortex and in the quantity and distribution of the lipoids (8, 10, 12, 17 *e.g.*).

Some experimental investigations, concerning the correlation between the intestinal obstruction and the histological picture of the adrenal cortex have been published. In these investigations the changes in the adrenal cortex caused mainly by the obstruction of the upper part of the intestinal tract are studied. A well demarcated zona glomerulosa and loss of lipoids from the zona fasciculata after 24—48 hours from the obstruction have been observed as clear changes (20). Furthermore, narrowing of the zona glomerulosa, diminution of the sudanophil material, haemorrhages and increased hyperemia of the zona fasciculata have been seen in duodenal obstructions of dogs (14, 15). Some clinical observations about the correlation between the intestinal disturbances and adrenal cortex have also been made, *f.ex.* the absorption disturbance from the duodenum observed in insufficiency of the adrenal cortex (6) and the loss of portions of the cords of cells, depletion of cortical lipids, cellular atrophy of the zona glomerularis and fasciculata and lymphocyte-like cell infiltrations of the adrenal cortex and medulla observed in tropical sprue (5). It seems, as if the pain should play a rather great role in the stress caused by a high intestinal obstruction, if we respect the anatomical situation, gastric secretion and the acute distension of duodenum or stomach. In the obstructions of lower part of the intestinal tract subjective symptoms and the disturbance in the fluid- and electrolyte balance are developing more slowly and one should expect that the possible morphological changes in the adrenal cortex should be rather mild, the adaptation time, thus, being longer.

The purpose of this study was to compare the manifestation of the non-specific stress caused by obstruction of the lower part of the intestinal tract to that caused by the obstruction of the upper part of the intestinal tract.

MATERIAL AND METHODS

The mechanical intestinal obstruction as a non-specific stress was studied by 62 male white rats and 12 controls. All rats were about 9 months of age. At the end of the experiment the rats were weighed. An aether narcosis was given to the rats and the gastrointestinal tract was ligated at different levels. In the first group of 28 rats the gastrointestinal tract was ligated from the pylorus.

In the second group of 10 rats the gastrointestinal tract was ligated from the lower part of the duodenum, at a distance of about 7 cm from the pylorus. In the third group of 10 rats the gastrointestinal tract was ligated from the lower end of the ileum. In the fourth group of 14 rats the gastrointestinal tract was ligated from the rectum, at a distance of about 3 cm from the anus. In the control group of 12 rats a laparotomy was made to the animals, the stomach and the intestines were taken out through the wound and then placed back again. The possible effect of these procedures, which were made also in the groups treated, on the weights of the adrenals could in this way be searched out. The animals were allowed to eat ad libitum. In each group the survival of the rats was controlled after 18, 24, 38 and 52 hours. At each moment of controlling the rats being in an agonal stage were killed by rapid decapitation. The last surviving rats were killed after 52 hours from the ligation. Immediately after killing the suprarenals were removed and weighed in a torsion balance. For study of the lipoid content of the adrenal cortex the left suprarenal gland was fixed for 24 hours in neutralized formol and 10 μ frozen sections were cut and stained by the Sudan III method. For more thoroughly morphological studies the right suprarenal gland was fixed for 5 hours in Zenker's solution and 5 μ paraffin sections were cut and stained by the van Gieson method and with haematoxylin and eosin.

RESULTS

The Survival Time of the Animals (Fig. 1). — Eighteen hours after ligation the mortality rate was 10.7 per cent in group I, in group II 10 per cent and in groups III, IV and in the control group 0 per cent. After 24 hours the mortality rate was 28.6 per cent in group I, in group II 11.1 per cent and in groups III and IV and in the controls 0 per cent respectively. In the cases examined after 38 and 52 hours the estimation of the mortality rate was disturbed by the fact that some animals were killed after 24 hours for autopsy. F.ex. in group I 8 animals were killed after 24 hours. From the remaining 12 rats 10 died, between the time from 24 to 38 hours, the mortality rate, thus, being as high as 83.3 per cent.

Findings on the Behaviour of the Animals during the Experiment and the Macroscopical Findings at Autopsy. — *Group I.* Already

Group	Hours after ligation				
	0	18	24	38	52
I	28	25 10.7 %	20 (8) 28.6 %	2 (1) 83.3 %	1 (1)
II	10	9 10.0 %	8 (7) 11.1 %	1 (1)	—
III	10	10 0 %	10 (4) 0 %	6 (4)	2 (2)
IV	14	14 0 %	14 (4) 0 %	10 (5)	5 (5)
Control	12	12 0 %	12 (4) 0 %	8 (4)	4 (4)

Fig. 1. — Influence of duration of obstruction on survival time. Mortality rate (%)

Upper value = number of animals alive

Value in parenthesis = number of animals killed

Percentage = mortality rate

after 18 hours from ligation of the pylorus the activity of the animals was decreased, they were soporous and they moved themselves slowly. After 24 hours they were standing in one place, the eyes half closed and breathing with a short amplitude. At autopsy the stomach was seen to be extremely distended by gas and undigested food.

Group II. As to the general condition of the animals and the appearance of the stomach the findings were about the same as in the preceding group, the part of the duodenum oral to the ligature also being very distended by undigested food and gas.

Group III. In this group the activity of the animals was seen to be somewhat decreased at first after 24 hours, the general condition, however, being, rather good. In the animals killed after 24 hours the small intestine oral to the ligation was about a thickness of a pencil. In the cases killed after 38 and 52 hours it was about a thickness of the little finger and distended by gas and fluid, the general condition of the animals being rather good.

Group IV. The development of the general condition was similar to the group III. In the animals killed after 24 hours the diameter of the sigmoid colon was a little larger than that of a pencil and in those killed after 38 and 52 hours it was nearly that of the little finger and the whole ileum a little distended.

The Mean Weights of the Adrenal Glands and their Weights mg./100 g. Body Weight (Fig. 2). — *Group I.* (Ligation at the pylorus).

Group	Control						I			II			III			IV		
	24	36	52	24	38	52	24	24	24	24	38	52	24	38	52	24	38	52
mg																		
Mean Weight of the Adrenals (+)	31.4	25.7	25.8	21.5	32.0	27.7	43.7	34.8	51.5	38.1	46.5	35.8	30.1	31.5	26.0	34.5	28.5	32.8
SE	± 1.95	± 0.76	± 0.88	± 1.31	± 2.01	± 1.43	± 2.77	± 3.06	± 0.05	± 0.05	± 0.05	± 0.05	± 0.05	± 0.05	± 0.05	± 0.05	± 0.05	± 0.05
P-value							0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Weight of the Adrenals (°)																		
60																		
50																		
40																		
30																		
20																		
10																		

Fig. 2. — The effect of ligation of the gastrointestinal tract on the weight of the adrenals of the male rat.

+ = The mean absolute weights of the adrenal glands
 ° = The weight of the adrenal glands mg/100 g body weight

The mean weights of the adrenals were 43.7 ± 2.77 , 51.5 and 46.5 mg. after 24, 38 and 52 hours respectively, and the average weights of the adrenals mg./100 g. Body Weight were 34.8 ± 3.06 , 38.1 and 35.8 in the animals killed after 24, 38 and 52 hours respectively; in the two last mentioned subgroups, respectively, only one animal had survived. It is observed, that the average weights of the adrenals mg./100 g. Body Weight in this group are higher than in the control group, the difference being statistically significant, except in the case examined after 52 hours. The mean absolute weights of the adrenals in the animals killed after 24, 38 and 52 hours were significantly larger than in the control group.

Group II. (Ligation at the duodenum). The mean weight of the adrenals was 35.8 ± 1.74 mg. in the animals killed after 24 hours and the average weight of the adrenals mg./100 g. Body Weight was 34.7 ± 1.38 . In this group the mean absolute weight of the adrenals is not significantly higher than in the control group, although the value mg./100 g. Body Weight is higher, the difference being statistically significant.

Group III. (Ligation at the ileum). The mean weights of the adrenals were 31.5 ± 1.78 , 34.5 ± 1.34 and 32.8 ± 0.75 mg., and the average weights of the adrenals mg./100 g. Body weight were 26.0 ± 1.56 , 28.5 ± 2.12 and 28.6 ± 0.92 in the animals killed after 24, 38 and 52 hours respectively. A statistically significant difference in the absolute weights and in the weights mg./100 g. Body weight of the adrenals, as compared to the control group, is observed only in the animals killed after 38 hours.

Group IV. (Ligation at the rectum.) The mean weights of the adrenals were 32.1 ± 1.78 , 32.8 ± 1.54 and 37.9 ± 1.11 mg. and the average weights of the adrenals mg./100 g. Body weight were 27.0 ± 1.66 , 29.7 ± 1.56 and 33.1 ± 0.92 in the animals killed after 24, 38 and 52 hours respectively. In the animals killed after 38 and 52 hours the difference between the average weights of the adrenals, as compared to the control group, was statistically significant both in the absolute weights and in the weights mg./100 g. Body weight.

Control Group. (Laparotomy). The mean weights of the adrenals were 31.4 ± 1.93 , 25.8 ± 0.88 and 32.0 ± 2.01 mg. and the average weights of the adrenals mg./100 g. Body weight were 25.7 ± 0.76 ,

21.5 ± 1.31 and 27.7 ± 1.43 in the animals killed after 24, 38 and 52 hours respectively.

Histological Findings in the Adrenal Cortex. — Control group. Sudan III: Plenty of small Sudan-positive droplets were seen in the cells of zona glomerulosa. The sudanophobic zone was clearly seen in all cases. In the cells of zona fasciculata rather large Sudan-positive droplets were seen, sudanophilia diminishing gradually towards the inner parts of the cortex and the zona reticularis being only weakly sudanophil. (Fig. 8). Van Gieson and hematoxylin-eosin: The zona glomerulosa was well formed, containing mainly small, moderately vacuolated cells. In all cases there was seen a transitional zone containing small cells with a more eosinophil and darker cytoplasm than the cells of the zona fasciculata. The cells of the zona fasciculata were stringly vacuolated, vacuolation diminishing gradually towards the inner parts of the cortex. In the cells of the zona reticularis a dense cytoplasm was seen with only some single, small secretion vacuoles. (Fig. 3). The same histological picture was seen in the cases killed after 38 and 52 hours.

Group I. (Ligation at the pylorus.) Sudan III: The zona glomerulosa was rather thin, its cells containing small Sudan — positive droplets. The sudanophobic zone was not to be seen in any case. Sudanophilia was strongest in the outer parts of the zona fasciculata, diminishing gradually towards the inner parts, zona reticularis being only weakly sudanophil. Van Gieson and haematoxylin-eosin: Zona glomerulosa was very thin, containing in some cases only two or three layers of cells. The transitional zone was not to be seen in any case. The cytoplasm in the cells of zona fasciculata was dense, containing only some few vacuoles. The sinusoids in zona reticularis were dilated (fig. 4). Necrotic foci and hemorrhages were seen in the zona fasciculata in some cases. No essential differences to the preceding cases were seen in the one being alive after 38 hours.

Group II. (Ligation at the lower part of the duodenum.) Sudan III: Sudanophilia and its distribution was in principle alike as in the preceding group (fig. 9). Van Gieson and haematoxylin-eosin: Zona glomerulosa was thin, containing only two or three layers of cells. No transitional zone was seen in any case. In the cells of zona fasciculata the vacuolation of the cytoplasm was only

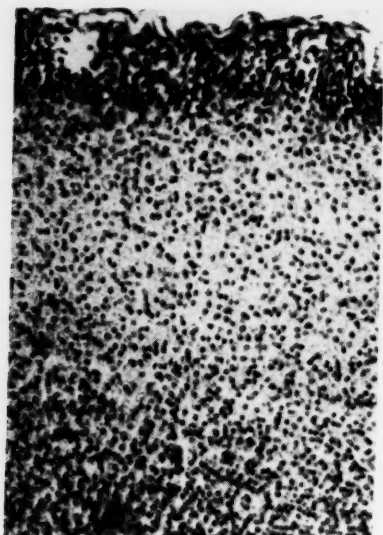


Fig. 3.

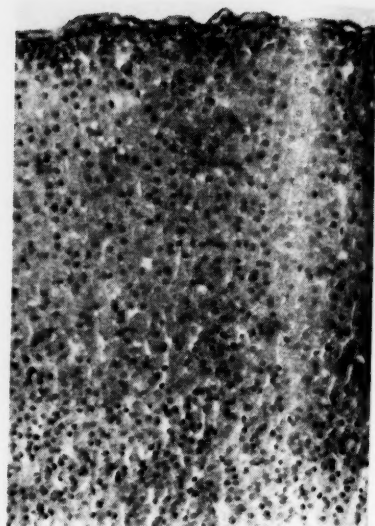


Fig. 4.

Fig. 3. — Photomicrograph showing adrenal cortex in the control group. Zona glomerulosa is well formed and the transitional zone clearly seen. 350 X, haematoxylin-eosin.

Fig. 4. — Photomicrograph showing adrenal cortex in the group in which the gastrointestinal tract was ligated at the pylorus. The glomerular zone is very small, the cells of zona fasciculata are dense and the sinusoids somewhat dilated. 350 X, haematoxylin-eosin.

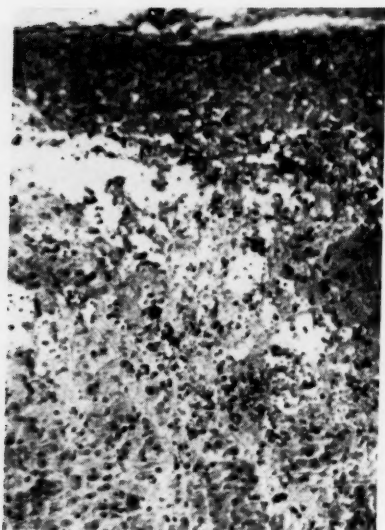


Fig. 5.

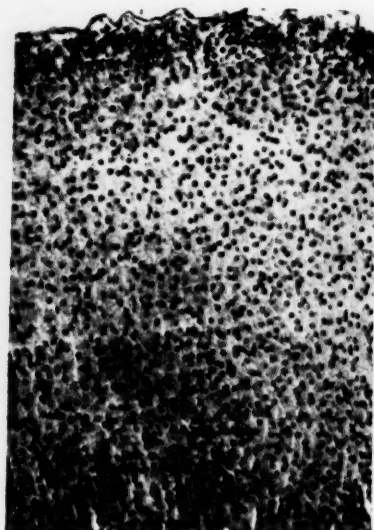


Fig. 6.

Fig. 5. — Photomicrograph showing adrenal cortex in the group in which the gastrointestinal tract was ligated at the lower part of the duodenum. A large necrotic and hemorrhagic area is seen. 350 X, haematoxylin-eosin.

Fig. 6. — Photomicrograph showing adrenal cortex in the group in which the gastrointestinal tract was ligated at the lower end of the ileum. An indistinct demarcation between the glomerular and fascicular zones is seen and a rather dense cytoplasm in the cells of zona fasciculata. 350 X, haematoxylin-eosin.

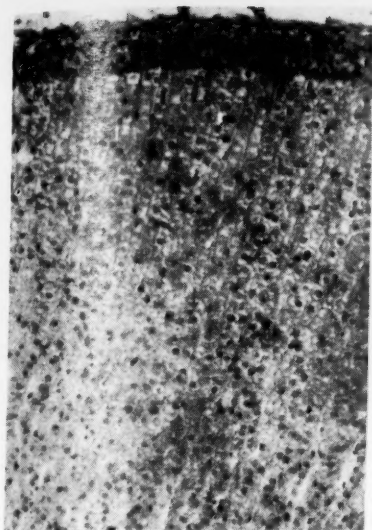


Fig. 7.

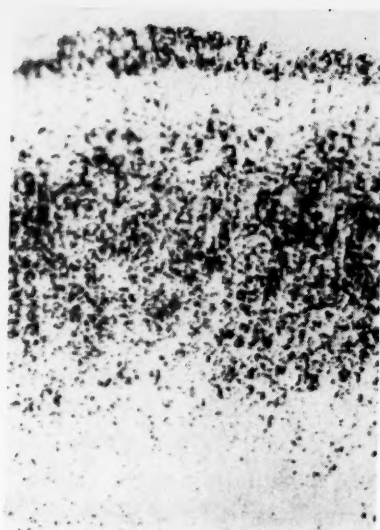


Fig. 8.

Fig. 7. — Photomicrograph showing adrenal cortex in the group in which the gastrointestinal tract was ligated at the rectum. A well formed zona glomerulosa is seen and its distinct demarcation from zona fasciculata. The cells of zona fasciculata are clear and vacuolated. 350 X, haematoxylin-eosin.

Fig. 8. — Photomicrograph showing sudanophilia of the adrenal cortex in the control group. The sudanophobic zone is well seen. 350 X, Sudan III.

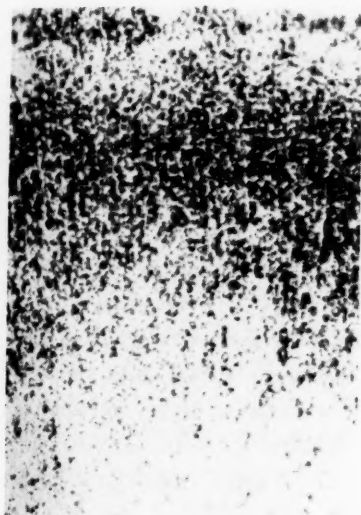


Fig. 9.

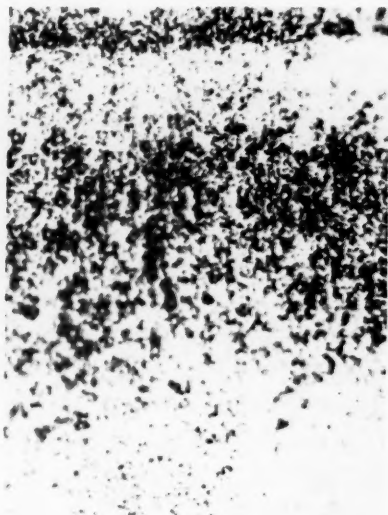


Fig. 10.

Fig. 9. — Photomicrograph showing sudanophilia of the adrenal cortex in the cases in which the gastrointestinal tract was ligated at the small intestine. Zona glomerulosa has diminished in width and the sudanophobic zone is indistinct. 350 X, Sudan III.

Fig. 10. — Photomicrograph showing sudanophilia of the adrenal cortex in the group in which the gastrointestinal tract was ligated at the rectum. No essential difference to the fig. 8 is seen. 350 X, Sudan III.

slight. Rather large necrotic and hemorrhagic areas were seen in zona fasciculata in some cases (fig. 5). In the whole cortex the sinusoids were dilated and blood-filled.

Group III. (Ligation at the lower part of the ileum.) Sudan III: Zona glomerulosa was rather thin, but clearly seen in all cases. The sudanophobic zone was weakly formed. The outer parts of zona fasciculata were stronger sudanophil than the inner parts (fig. 9). Van Gieson and haematoxylin-eosin: Zona glomerulosa was of a thickness of about three or four layers of cells but the demarcation line between the glomerular and fascicular zones was indistinct. The vacuolation of the cells of zona fasciculata was still relatively poor. Necrotic and hemorrhagic foci were sometimes seen in zona fasciculata. The sinusoids were dilated, especially in the zona reticularis (fig. 6). In the cases being alive after 38 and 52 hours no essential difference to the preceding cases was seen in the histological picture.

Group IV. (Ligation at the rectum, about 3 cm from the anus). Sudan III: Zona glomerulosa was well formed and the sudanophobic zone was seen in most cases. Sudanophilia was rather strong in zona fasciculata, stronger in its outer than in the inner parts (Fig. 10). Van Gieson and haematoxylin-eosin: The morphological picture was in principle the same than in the control group. Zona glomerulosa was well formed, of a thickness of several layers of cells. The vacuolation in the cells of zona fasciculata was moderate. The transitional zone was seen in some cases, in other cases the transition between the glomerular and fascicular zones was abrupt and the demarcation line very distinct (Fig. 7). The cells of zona reticularis were sometimes very large and filled by colloidal particles.

The microscopical findings were nearly alike these in the cases killed after 52 hours, but in the cases killed after 38 hours still some other changes were seen. In these cases zona glomerulosa was very thin and the cell cords of zona fasciculata seemed to continue until the capsule of the gland. In some cells of zona fasciculata colloid droplets were seen. No transitional zone was to be seen. The vacuolation in the cells of zona fasciculata was relatively poor as compared to the control group.

DISCUSSION

A tendency to a rapid shock and depletion of the general condition in high intestinal obstructions is believed to be caused mainly by rapidly developing metabolic disturbances and of gas accumulating in the stomach (4, 18), and this in turn has come from swallowed air and from the digestion (18). In the lower obstructions the disturbances manifest themselves more slowly. The role of the inflammatory component is evidenced by the prolongation of the survival time of the rat after administration of antibiotics to the intestine (19).

The surgical stress in abdominal operations manifest itself in the function of the adrenal cortex through rise of the 17-hydroxycorticosteroids in the blood plasma after operation (16). In cytochemical studies in a surgical stress it has been observed an unchanged content of the desoxyribonucleic acid in the adrenal medulla, the tyrosine content increasing clearly in the same time. This has been observed in subtotal gastrectomies in dogs (3). The mechanical intestinal obstruction is apparently a rather strong non-specific stress which is evident from the clear changes observed by us in the weights of the adrenals and in the histological picture of the adrenal cortex. The changes are partly affected through the depression of the general condition of the animals, partly through the continuous increase of the stress.

The hypertrophy of the adrenal cortex, which manifests itself in the histological picture and in the increase of the weights of the adrenals, is one of the morphological manifestations of a non-specific stress (8, 10, 17). In our series the increase of the weights of the adrenals was especially prominent in group I. As compared to the control group it was interesting to note a statistically significant increase of the weights of the adrenals also in the group IV after 38 and 52 hours from the ligation. This is apparently caused by a later developing stress in this group, which is in accordance with the clinical picture and the later developing symptoms. The necrotic and hemorrhagic foci observed in the adrenal cortex in groups I and II as a sign of an acute and intense stress are in accordance with earlier findings (15). Reports regarding morphological changes in the adrenal cortex in an obstruction of the small intestine are scanty. A diminution of the nuclear volume, however,

has been found in karyometric studies (7). Although the changes caused by obstruction of the lower intestinal tract are slight, it is interesting to note a histological reaction in the adrenal cortex after 38 hours. This reaction manifests itself in the diminution of the width of zona glomerulosa, disappearance of the transitional zone and diminution of the secretion vacuoles in the cells of zona fasciculata. This stress is probably effected through the increase of the gas content of the intestine, impossibility of defecation and through pain. It is remarkable, however, that the only manifestation of stress in the cases killed after 52 hours was the increase of the weights of the adrenals. In obstructions of the upper part of the gastrointestinal tract a large proportion of nitrogen has been found in the gas content of the intestine, which in turn is caused by swallowed air (18). It appears as if the changes in the adrenal cortex were partly caused by a disturbed fluid balance, because in duodenal obstructions dogs have maintained a longer time in good general condition through administration of fluids and this treatment has been found to have a beneficial influence upon the histological picture of the adrenal cortex (20). Also a parenteral administration of suprarenal extracts has prolonged the survival time of experimental animals. This, however, has not had any effect on the histological picture of the adrenal cortex (15). It is evident that the tendency to shock, depression of the general condition and histological signs of stress in the adrenal cortex are more prominent in obstructions of the upper part of the gastrointestinal tract, less prominent in obstructions of the lower part, although a clear reaction in the adrenal cortex can be seen in obstructions as low as at the communication between rectum and sigmoid colon.

SUMMARY

The effect of a mechanical abdominal stress on the adrenal cortex was studied by ligating the gastrointestinal tract of 62 male white rats at different levels. 12 animals served as controls.

In the group in which the ligation was made at the pylorus the mortality rate after 24 hours was 28.6 per cent. The weights of the adrenals had increased significantly, except in the case examined after 52 hours. In the histological picture of the adrenal cortex signs

stress were observed in form of some necrotic and hemorrhagic of foci, diminution of the width of the zona glomerulosa, disappearance of the transitional zone and diminution of the secretion vacuoles in the cells of zona fasciculata.

In the group in which the ligation was made at the lower part of the duodenum the mortality rate after 24 hours was 11.1 per cent. The increase of the weights of the adrenals was not as prominent as in the preceding group but the histological signs of stress in the adrenal cortex were about the same as in the preceding group.

In the group in which the ligation was made at the lower end of the ileum the weights of the adrenals did not increase significantly and the mortality rate was 0 per cent after 24 hours. The general condition of the animals after 52 hours was rather good. The histological changes in the adrenal cortex were milder than in the preceding groups.

In the group in which the ligation was made at the rectum the mortality rate and general condition of the animals were as good as in the preceding group. The weights of the adrenals increased significantly after 38 and 52 hours, but only in the cases examined after 38 hours mild signs of stress were present in the histological picture of the adrenal cortex.

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EFFECT OF PROCHLORPERAZINE (STEMETIL) ON THE RAT'S AREA POSTREMA

A HISTOCHEMICAL STUDY

by

I. MÄKINEN, E. KIVALO, and J. TYRKKÖ

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For a number of years, phenothiazine derivatives have been used successfully in clinical work for the treatment of a great variety of diseases. The suitability of these preparations for medication of the most widely different range of symptoms is a consequence of their great number of active components. Among the peripheral effects of the phenothiazine derivatives, the following may be mentioned here: spasmolytic, adrenolytic, slightly ganglion-blocking, anticholinergic, and local anaesthetic effects. As a consequence of central action, again, antispastic, hypotonic, analgetic, hypothermal and antiemetic effects, among others, are obtained with these substances. The different effects are somewhat different in strength for various substances of this group, depending on their chemical constitution (11).

Prochlorperazine has been noted to possess an antiemetic effect about five times that of chlorpromazine and less strong secondary effects than the latter (10). For instance, prochlorperazine causes less hypotonia, and no disturbances of the liver have been observed in connection with its use (5,9). The substance has therefore become widely used as an antiemetic although it is also encountered in use in other indication regions, in likeness to the other phenothiazine derivatives (10, 14, 17).

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The present work is an attempt to find out with the aid of certain histochemical methods whether any changes can be demonstrated in the rat's area postrema upon administration of prochlorperazine. After Borison and Wang, in 1948 (2), found that the vomiting centre is situated in the brain stem, *i.e.*, in the reticular formation of the medulla oblongata, the area postrema has been considered to constitute the site of this centre (3, 13). In this area, which is situated in the medulla oblongata, on the floor of the fourth ventricle, substance P has been found to occur in exceedingly greater quantity than anywhere else in the brain stem, its profusion being comparable with the concentration of the same substance in the mesencephalon (1). Furthermore, serotonin has been found in exceptional abundance in the area postrema (1), and sympathin in nearly equal quantity as in the hypothalamus (18). Histochemically, comparatively strong acid and alkaline phosphatase activity has been established; the same applies to non-specific esterase and succinic dehydrogenase activity (8). In the opinion of Leduc and Wislocki, this would indicate, *e.g.*, that this part of the brain can be compared with the glandular tissues elsewhere in the body although no distinct secretive activity is observable in it (8).

In likeness with the other phenothiazine derivatives, prochlorperazine exerts an influence upon the brain stem, that is, upon its centrencephalic system; its antiemetic effect, for instance, is made understandable by one action being on the chemoreceptor trigger zone, the area postrema, in the medulla (10). In the present work an attempt was made to locate histochemically the potential effect of prochlorperazine on the area postrema, employing as indicators the acid phosphatase activity and succinic dehydrogenase activity, which are involved in the protein synthesis and are directly proportional to the strength of the metabolic processes in the tissue (8, 12).

MATERIAL AND METHODS

Altogether 40 adult white rats, both male and female, were used in this work. Twelve rats were injected intraperitoneally with prochlorperazine (Stemetil) at a dosage of 10 mg per kg body weight, which was sufficient to make the animals slightly somno-

lent. Eight rats were given apomorphine subcutaneously 10 mg per kg body weight; the rats seemed to tolerate this dose quite well. No vomiting occurred but the animals were clearly nauseated. Twenty rats served as controls. The experimental animals were killed by rapid decapitation without anaesthesia, two hours after the prochlorperazine injection or 20—30 minutes after the apomorphine injection. The medulla oblongata was immediately laid bare and excised for investigation.

1) Demonstration of phosphatase was done by Eränkő's (6) modification. The cuts had a thickness of 20 micra and were incubated for two hours.

2) For the histochemical demonstration of succinic dehydrogenase, the medulla oblongata was immediately frozen and sliced transversally on the ice microtome into cuts of 30 micra, which were incubated in accordance with the method of Seligman and Rutenburg (15), using neotetrazolium for the succinic dehydrogenase indicator. However, the incubation process was different from the above-mentioned method, in that it was carried out relatively anaerobically in closed flasks.

RESULTS AND DISCUSSION

On comparing the area postrema of each experimental animal and that of the control animal sacrificed at the same time the following observations were made.

In the said brain tissue of all controls normal acid phosphatase activity and succinic dehydrogenase activity could be demonstrated in conformity with the descriptions found in the literature (8, 16). The administration of apomorphine to the test animals was intended to stimulate their area postrema, since this substance is known to excite the vomiting centre already in small doses (4). However, neither enzyme system revealed any significant change. This may be taken to indicate that apomorphine exerts an influence on the vomiting centre of the medulla itself rather than on its chemoreceptors.

Upon administration of prochlorperazine, on the other hand, the findings were in the first place that acid phosphatase activity (normally evident in very great strength in the nuclei of the parenchymal cells of the area postrema and in moderate strength in

their cytoplasm) was clearly reduced in the nuclei and also slightly lower in the region of the cytoplasm. With reference to the surrounding brain tissue, the area postrema of prochlorperazine-medicated rats displayed distinct diminishing of enzymatic activity; for instance, no change as compared to the controls could be noted in the ganglion cells of the nucleus of the nervus hypoglossus in this respect (Figs. 1 and 2). On the other hand it has to be noted



Fig. 1. — The hypoglossal nucleus and area postrema of the normal rat show marked acid phosphatase activity. \times about 70.



Fig. 2. — The area postrema of prochlorperazine-medicated rat display distinct diminishing of acid phosphatase activity. \times about 70.



Fig. 3. — Succinic dehydrogenase activity of the normal rat's area postrema. Note moderate activity in the area postrema and marked activity in the hypoglossal nucleus. \times about 50.

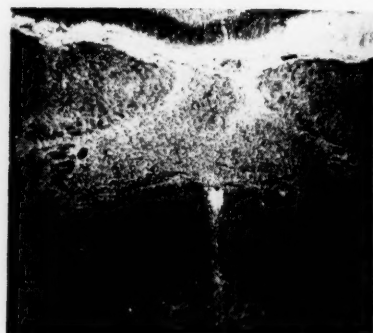


Fig. 4. — Succinic dehydrogenase activity of prochlorperazine-medicated rat. An evident activity is exhibited as in fig. 3 in the hypoglossal nucleus, little if any activity in the area postrema. \times about 50.

that the acid phosphatase activity in the ganglion cells of the nucleus of the nervus vagus beside the area postrema was, perhaps, slightly lowered.

In exactly the same manner, distinct decrease in the distribution of the formazan crystals indicating succinic dehydrogenase activity could be observed, upon prochlorperazine administration, in the entire region of the area postrema, as compared with the controls. This, too, could also be demonstrated by comparison of the area with its surrounding tissues (Figs. 3 and 4), as in the case of the acid phosphatase.

Examination of the nuclei of the eighth cerebral nerve, in the respect mentioned above, revealed no change in the rats treated with prochlorperazine, as compared with the controls.

From the results presented here, the inference can be drawn that the effect exerted by prochlorperazine on the centrencephalic system of the brain stem in general does not manifest itself, at least not in the region of the medulla oblongata, as lowering of the said enzymatic activities anywhere else but in the region of the area postrema. This is thought to indicate that prochlorperazine is specially capable of deprimating the metabolism in the cells of this particular part of the brain, its strong antiemetic effect being a consequence of this action.

SUMMARY

The effect of prochlorperazine (Stemetil) upon the site of the chemoreceptors of the vomiting centre in the rat's medulla oblongata, the area postrema, has been studied. Acid phosphatase and succinic dehydrogenase activity were used as indicators. Comparison between the test animals and controls showed that distinct reduction of the said enzymatic activities in the area postrema was produced by prochlorperazine. On the other hand this activity was unchanged in the surrounding brain tissue, in particular in the ganglion cells of the nuclei of n. hypoglossus, n. vagus and n. statoacusticus. This result is thought to indicate that prochlorperazine has a particularly strong effect on the area postrema and that this may be the basis of its antiemetic action.

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EFFECT OF NORADRENALIN ON THE MYOCARDIUM OF RAT

by

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(Received for publication July 19, 1960)

During the past few years there have been numerous investigations on the effects of noradrenalin on the circulatory system and especially on the heart. Some of them deal also with the histological picture of the heart.

It has been found that noradrenalin affects the heart in many respects similarly to sympathetic nervous excitation. This indicates that noradrenalin is the principal sympathicomimetic agent of the heart. When the accelerator nerves of the dog were stimulated noradrenalin mainly was secreted in coronary blood and that the amount of adrenalin, if any, did not exceed 3 per cent (8). Dilatation of the coronary vessels of dog under the influence of noradrenalin has been observed (4) as well as an increase in coronary circulation (12, 13).

On the other hand, it has also been claimed that noradrenalin causes coronary constriction and that dilatation is a secondary condition, attributable, at least in part, to peripheral effect. The influence of the metabolic products should also be taken into consideration (2). In performing tests on the hearts of human fetuses a coronary constriction was produced by noradrenalin and adrenalin in 7 out of 8 cases (1). The coronary flow also in rat heart diminished under the influence of noradrenalin (6). An increased noradrenalin and adrenalin concentration in the coronary circulation also raises the quantity of lactic acid which in turn brings about a proportionate

* Aided by a grant from the Sigrid Jusélius Stiftelse.

increase in the oxygen consumption (10). This results in sporadic anoxia in the cardiac muscle, which may cause pathological changes. A profuse infiltration of fatty droplets in the myocardial fibrils of the dog given a massive noradrenalin and adrenalin infusion was present when the animals were killed on the day following the infusion (7). If they were killed 3 or 5 days later, or had been given Dibenzylamine prior to the infusion, the changes were milder. Sporadic myocardial lesions in rats with subcutaneous noradrenalin injections has been found (11). If corticoids were administered with the noradrenalin injections the lesions were considerably greater. Raab, too, refers (9) to the histological changes induced by sympathetic nerve stimulation, *e.g.* cardiac hypertrophy, degenerative changes of the myocardium and coronary sclerosis.

As seen above many workers have arrived at differing results concerning the effect of noradrenalin on the heart. As the observation time has usually been short in these investigations, the present study was partly undertaken to establish the effect on the histology of the cardiac muscle of noradrenalin injections administered over a longer period.

MATERIAL AND METHODS

The series studied consisted of 28 white male rats. L-noradrenalin injections were administered daily to 4 groups of 5 animals each. The amount injected was 0.4 mg. The remainder of the test animals constituted the control group. The first test group was killed 2 weeks from the beginning of the experiment the others at 2-week intervals, the last group after 8 weeks. The hearts were weighed and fixed in 10 per cent formalin solution. Staining was performed with hematoxylin-eosin.

RESULTS

No changes were observed in the hearts on gross examination. The heart weights were calculated per 100 g of body weight. The mean heart weight increased initially during the experiment. In animals killed after 2 weeks the heart weighed 348.3 mg/100 g, after 4 weeks 365.3 mg/100 g, after 6 weeks 378.5 mg/100 g. The weight was lower, 334.4 mg/100 g, for the animals treated for 8 weeks. It was nearly the same as with the controls for which it was 334.0 mg/100 g.



Fig. 1. — Photomicrograph of a rat heart of the control group, $\times 540$.

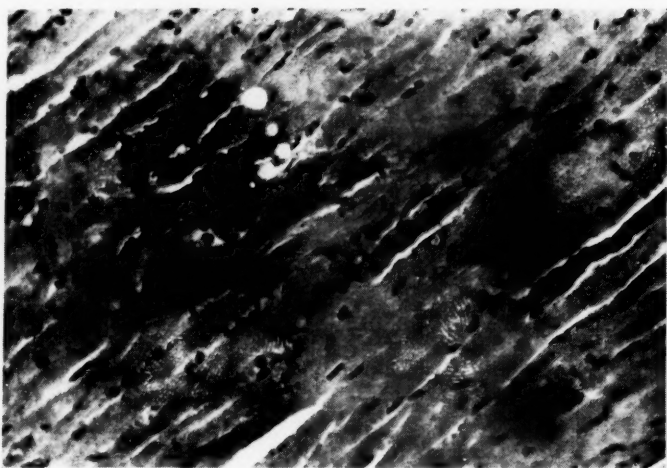


Fig. 2. — Photomicrograph of a rat heart after 6 weeks' treatment. Staining of muscle fibres uneven, $\times 240$.

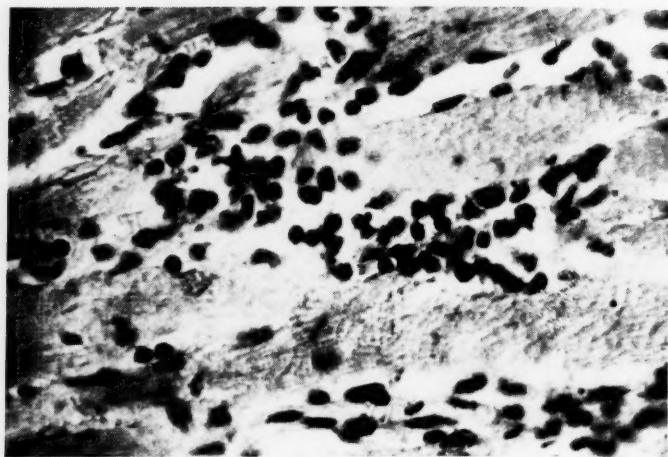


Fig. 3. — Rat heart after treatment of 6 weeks. Muscle fibrils are swollen, detailed structure disorganised. Cellular infiltration, $\times 540$.

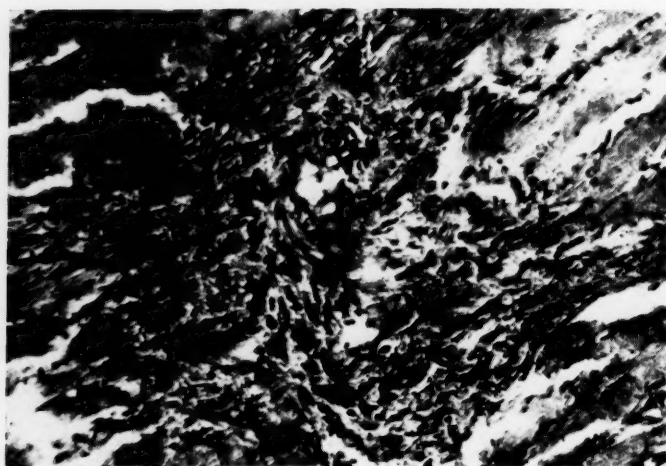


Fig. 4. — Rat heart after an 8-week treatment. Proliferation of connective tissue, $\times 240$.

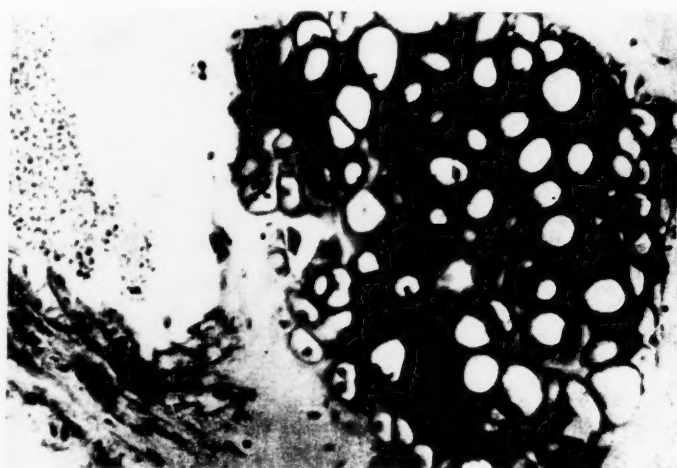


Fig. 5. — Rat heart after an 8-week treatment. Chondrogenesis near the aortic valve, $\times 240$.

Microscopic examination revealed hardly any difference from the controls in the hearts of the animals given L-noradrenalin for a shorter period, 2 and 4 weeks. Staining was perhaps slightly more uneven in places. On the other hand, a few animals in the 6-week group and the majority in the 8-week group showed swollen muscle fibrils in some places in the ventricle wall. The crossstriation had become more indistinct and the muscle looked like a homogeneous mass. The number of the nuclei seemed to be reduced in the corresponding places. Sporadic leucocyte infiltration was also observed. The muscle fibrils of a couple of the test animals in the last-mentioned group appeared to be broken and there was connective tissue proliferation between them. In the hearts of the animals subjected to the longest treatment displayed profuse formation of cartilaginous tissue near the aortic valves, and the wall of the aorta bulged towards the lumen. A few cases also showed cartilage in the ventricular septa.

DISCUSSION

Subcutaneous injections of noradrenalin provoked distinct changes in rats after treatment lasting for 6 and 8 weeks respectively. After the shorter period staining was sometimes uneven

in places, some muscle fibres staining more heavily than their environment. The effect of a single injection appeared to be mild, and only repeated injections for a sufficiently long time produced observable changes.

It is difficult to explain the causes of the present results. According to Raab (10), catechol amines, thus also noradrenalin, accelerate the metabolic function. If in the present case, noradrenalin induced coronary constriction, degenerative changes due to ischemia might be caused. An ischemia originating in this way has been assumed to cause a fatty degeneration of muscle fibrils in dogs (7). In this work the proliferation of connective tissue established in places in the myocardium is obviously scar tissue. Hyaline degeneration, necrosis and cicatrization have been seen in patients receiving massive doses of adrenalin for asthma over a longer period (3). The daily handling of the rats and the injections they received in the present investigation constituted perhaps stress enough to aggravate the changes like the concurrent administration of noradrenalin and corticoids (11).

As regards the changes occurring in heart weight, similar results have been obtained with adrenalin injections administered over a longer period of time (5); in other words the relative weight of the hearts approached that of the controls the longer the test lasted. It is probably a question of adaptation. An adaptation to the effect of adrenalin can be observed in rats after more than 10 days of adrenalin treatment (14).

It is difficult to explain the correlation between the profuse cartilage formation observed in the aortic wall and noradrenalin treatment.

Long term treatment with noradrenalin injections is obviously capable of inducing changes in the histological structure of the myocardium. No definite reason for the changes has been offered but it seems that metabolic alterations and possible vasoconstrictions have a prominent role in their genesis.

SUMMARY

Noradrenalin was injected subcutaneously into male rats in a daily dosage of 0.4 mg. Short-term treatment provoked no notable changes in the histological structure of the myocardium. When

the injections were continued for 6 and 8 weeks the following observations were made: the muscle fibrils stained unevenly in places, there was swelling and cross-striation disappeared the tissue resembled a homogeneous mass. Obvious scar tissue formation was also seen in some places. Profuse chondrogenesis was demonstrated near the aortic valves.

The relative weights of the hearts increased initially, but towards the end of the experiment they were reduced near normal values again.

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EFFECT OF BENZYLIMIDAZOLIN UNDER AUDIOGENIC-VISUAL STRESS CONDITIONS

by

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Several investigators have noticed that different stress conditions produce organic or functional changes in the reproductive organs of test animals. According to Selye (9) certain stress stimuli bring about ovarian atrophy. It has been found that in rats after light, sound and electrical stimuli there occurred first the acceleration of the oestrus cycle and thereafter hypofunction and inactivity of the ovaries, shown by continuous dioestrus (1). Audiogenic-visual and painful stimuli produced in rats a decrease in the weight of the ovaries and the pituitary glands and a reduction of gonadal functions (10). Testis atrophy was observed in stressed rats by Jänkälä and Näätänen (7). Similar changes have also been found in man (12, 14).

The ovarian changes would be caused in two different ways: through the nervous system and through the cortico-hypothalamo-hypophysis (1). Hypothalamus is an intermediary centre between external stimuli and various organs; it receives the cortical stimuli from external origin (optical, acoustical, thermic, sensations of pain, etc.), transforms them and mediates them to different organs either through the vegetative nervous system or through the hypophysis. Referring to the anterior lobe of the hypophysis Selye (9) mentions the decreased secretion of gonadotrophins. The decreased gonadal function with gonadal atrophy in prolonged stress is attributable to a changed production of gonadotrophins (10).

¹ Aided by a grant from the Sigrid Jusélius Stiftelse.

Emotional stimuli are said to increase adrenergic hormone secretion and thus to cause vasoconstriction (3). According to Selye (9) the adrenergic substance would be liberated by the hypothalamo-autonomic nervous system-adrenal medulla. Stress might also effect the posterior lobe of the hypophysis thus causing the secretion of vasopressin. It has been observed that psychic strain can increase the secretion of adrenalin many times over normal in man (8). A tendency to elevated blood pressure was observable in stressed pregnant rats (11).

The presence of vasoconstrictive agents seems to be a characteristic of different stress conditions. The object of the present study is to investigate whether a vasodilative sympathicolytic substance commands an antagonistic effect on certain changes occurring under stress conditions. Special attention will be paid to ovaries.

MATERIAL AND METHODS

Benzylimidazolin (Priscol)¹ is a sympathicolytic substance with a direct effect on the local vessels, dilatation occurring within 10 min. after an intramuscular injection (2). The dilatory effect is observed particularly in arterioles and in small arteries (13). Elevated blood pressure produced by adrenalin was eliminated by this substance in rats (4) and in guinea pigs (5).

The material consists of 63 virgin female rats at the age of 5 months and with the average weight of 179 gm. The actual test series included four groups:

Group a, which was given benzylimidazolin (Priscol, Ciba) 1.5 mg subcutaneously twice a day for each rat.

Group b, which underwent an audiogenic-visual stress treatment. It was largely similar to that used by Soiva *et al.* (10); yet no pain stimuli were made use of at all. The treatment was given twice a day for 1.5 hours at a time. One hour before the treatment, benzylimidazolin was injected as mentioned in connection with group a.

Group c, which underwent the stress treatment described above; yet no injections were given.

Group d, which was used as a control group and allowed to live under normal laboratory conditions.

¹ Kindly supplied by Ciba.

After a 40 days' treatment the animals were weighed and killed by means of decapitation. The adrenals, hypophyses and ovaries of both the test animals and the controls were prepared and weighed. For microscopic studies the ovaries were fixed in neutral 10% formalin for 24 hours, dehydration in the usual way and embedded in paraffin. They were cut in five μ slices and stained according to van Gieson. The number of primary, Graafian and atretic follicles and of normal corpora lutea was determined for each ovary using six slices.

RESULTS

Weight of the Animals and the Organs (Table 1). — The mean final body weights of the animals in different groups did not differ

TABLE 1

Group	Number of Animals	Body weight-gm		Adrenals	Hypophysis	Ovaries
		Initial	Final	mg	mg	mg
a	16	179	207 \pm 6.8	61.5 \pm 2.1	11.2 \pm 0.5	106.8 \pm 4.1
b	16	179	204 \pm 5.6	67.0 \pm 2.7 ¹	10.6 \pm 0.3	105.6 \pm 3.8
c	15	179	199 \pm 6.7	65.5 \pm 2.9 ¹	10.1 \pm 0.5	104.0 \pm 4.1
d	16	179	205 \pm 4.7	55.8 \pm 2.8	11.0 \pm 0.3	104.8 \pm 3.5

¹ The difference between the means was considered significant when P was \leq 0.05.

significantly from each other; the increase in weight appears to be smallest in group c.

In test groups b and c the average weight of the adrenals was significantly greater than that of the control group ($P < 0.01$ and 0.05).

No significant differences could be observed in the weight of either the hypophyses or the ovaries between different groups, although the lowest one of both the hypophyses and ovaries seemed to be in the group c.

Changes in the Histologic Features of the Ovaries (Table 2). — In the stress group and in the stress group that had received benzylimidazolin the mean number of primary, Graafian and atretic follicles and that of corpora lutea were largely similar. As compared with the control series it was observed that the number of primary follicles in the stressed group was significantly smaller, and that

TABLE 2

Group	Primary Follicles	Graafian Follicles	Atretic Follicles	Corpora Lutea
a	1.5 ± 0.3	6.1 ± 0.5	6.3 ± 0.7	9.2 ± 1.7
b	1.1 ± 0.1	6.0 ± 0.9	8.8 ± 0.8	12.5 ± 1.7^1
c	0.9 ± 0.2^1	7.2 ± 0.7^1	8.3 ± 0.7	12.3 ± 1.4^1
d	2.0 ± 0.5	4.3 ± 0.8	7.0 ± 0.6	8.7 ± 0.7

¹ The difference between the means was considered significant when P was ≤ 0.05 .

of Graafian follicles, significantly greater. The number of corpora lutea was significantly greater in both the stressed group and the group treated with benzylimidazolin as compared with the controls.

DISCUSSION

The changes in test animals produced by the stress treatment were not so evident in the present material as those reported before (1, 10). One probable explanation for this fact can be found in the intensity of the treatment; in the present study the test animals were only subjected to audiogenic-visual stimuli, no pain stimuli being made use of. The effectiveness of the treatment is, however, proved by the fact that the adrenals were significantly enlarged in groups b and c.

No significant differences can be seen between the mean weights of ovaries in the different groups. In the stress group, however, there seems to be a slight tendency to a decrease in weight. The number of primary, Graafian and atretic follicles and that of corpora lutea are quite similar in groups b and c on one hand and in groups a and d on the other hand. As compared with the control group there are to be seen significant differences with respect to primary follicles, Graafian follicles and corpora lutea in the stress group and also respect to corpora lutea in the stress group that received benzylimidazolin. The changes similar to those has been reported before (10). A contradictory result is furnished by the large number of Graafian follicles in the present work. The difference in the intensity of the stress may be considered a probable cause for this fact.

Benzylimidazolin was not observed to have any definitely antagonistic effects on the changes produced by stress. Apparently

vasoconstriction is not the decisive factor in the occurrence of such changes. The substance itself does not seem to cause changes in the weight of the ovaries, hypophyses and adrenals or in the microscopic picture of the ovaries either. The test subjects of the present study having been rats, the results need not necessarily be similar as regards other test animals or man.

SUMMARY

The writers tried to find out whether benzyylimidazolin has prohibitory effects on certain changes occurring in test animals under strain conditions. The series consisted of 63 female virgin albino rats, which were at the age of five months. The weight of the adrenals was significantly increased both in the stress group and in the stress group that had been given benzyylimidazolin. No significant differences existed between the mean weights of the hypophyses and ovaries in the different groups. The microscopic structure of the ovaries in the test group under stress treatment was largely similar to that seen in the stress group that received benzyylimidazolin. The control group differed quite clearly from the two in this respect. The substance being investigated had no effects on the changes by itself.

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ISOLATION OF A PARTICULATE FRACTION FROM PIG AORTA

by

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(Received for publication July 22, 1960)

The formation of atheromatous lesions in the human aortic tissue in arteriosclerosis is a phenomenon which is not yet well understood. Many theories concerning the pathogenesis of this disease have been given. Some postulate a passive imbibition of lipides from the blood into the intimal layer of the blood vessel, some consider a metabolic change in the arterial wall to be the basic cause to the lesions.

In view of the latter hypothesis, that the primary lesion would be a metabolic deterioration in the arterial wall, it is surprising how little is actually known about the metabolism of the tissue in question. This is probably due to the great technical difficulties encountered in such studies. Some studies, mainly concerned with the oxygen uptake of intact aortic tissue, have appeared (2, 10). Chernick *et al.* (7) studied the lipide synthesis in the aorta, and Zilversmit (15) has indicated that phospholipides may play a part in the formation of the atherosclerotic lesion. The sulfate metabolism of the aortic wall was studied by Boström and Odeblad (1, 14) and by Hilz and Utermann (11). Furthermore in a series of papers Buddecke (3—6) reports extensive analytical studies on human aortic tissue. He found considerable differences in the composition of the normal and arteriosclerotic aorta. Especially an increase in the acid mucopolysaccharides of the heparin type seemed to be significant in the arteriosclerotic process.

The isolation of enzymatically active entities from aortic tissue could be of great help in the further investigation of metabolic processes involved in the pathogenesis of arteriosclerosis. The present paper describes the preparation of a homogeneous particulate fraction from pig aorta. The lipide composition of this fraction has been studied and compared with that of the intact aorta. In a subsequent paper (12) some metabolic properties of this fraction will be reported.

ANALYTICAL METHODS

Dry weights were determined by drying the samples to be analyzed in small beakers in an oven at 95°C, after which they were placed in a desiccator and weighed when cool. In the case of the particulate fraction II, the sucrose in the suspension medium was removed by mixing the sample with a tenfold volume of distilled water, and centrifuging at 40,000 rpm. in the Spinco Model L centrifuge. The washing procedure was repeated once. Additional washings did not change the dry weight of the sample.

Total Lipids. — The procedure of Folch *et al.* (9) was used for extraction of lipides. The lipids, which appear in the lower phase solvent, were determined by weighing the dried extract.

Cholesterol. — This was determined from the lipide extracts dissolved in chloroform with the usual Liebermann-Burchard procedure as described by Nikkilä (13).

Lipide Phosphorus. — Lipide phosphorus in the extract was determined according to Fiske and Subbarow (8). Instead of the usual aminonaphtholsulfonic acid reagent, 2,4-diaminophenol hydrochloride (Amidol) was used as reducing agent. The phosphorus values were converted to phospholipid values using the factor 25.

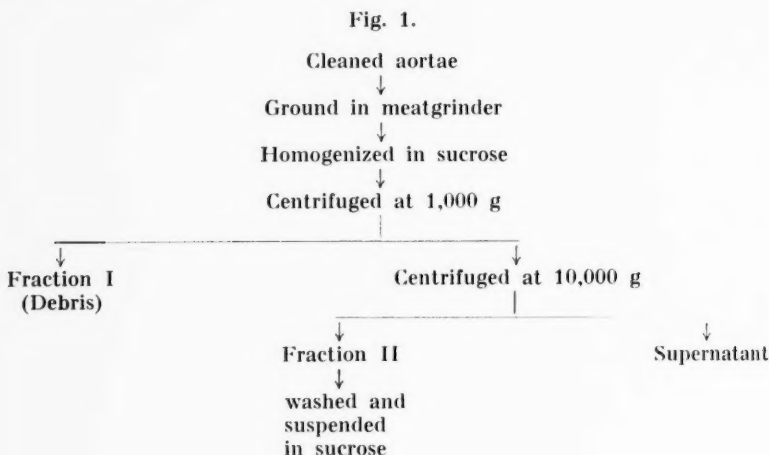
PREPARATIVE PROCEDURE

The pig aortae were obtained from the municipal slaughterhouse in Helsinki. The aortae were removed from the carcass and collected in ice prepared from an isotonic sucrose solution. This usually took place about 15 minutes after the killing and bleeding of the animal. For one days experiments it was usually sufficient to obtain aortae from 30 to 40 pigs. Only the thoracic partion of the

aorta was used for experiments. When the material arrived at the laboratory, all loose fat and connective tissue on the outside of the aorta was carefully removed, care being taken not to warm up the tissue excessively during this process. The cleaned and fat-free blood vessels were placed again on sucrose-ice. The subsequent operations were performed in the cold room.

The tissue was ground twice in a precooled electric meatgrinder. 200 gms of the ground aortamine was then blended together with 400 ml of 0.25 M sucrose for 20 seconds in a high speed homogenizer. The homogenate so obtained was centrifuged at 1,000 g for 2 minutes, after which the supernatant was poured off and filtered through cheesecloth. The cloudy supernatant was centrifuged at 10,000 g for 15 minutes (10,000 rpm. in the No. 20 rotor) in the Spinco model L ultracentrifuge. The residue was collected, suspended in 0.25 M sucrose with 1 mM $MgCl_2$ and centrifuged again at 10,000 g for 10 minutes (13,000 rpm. in the No. 40 rotor). The final residue was suspended in about twice its own volume of 0.25 M sucrose. In some cases, where the main purpose of the isolation was the determination of the lipids of the particulate fraction, it was suspended in 0.88 M sucrose, which effectively prevented clumping of the material upon freezing and thawing. The material could thus be stored frozen for several days.

The fractionation procedure is illustrated in *Fig. 1*. Fraction I is the low speed residue, which is discarded, fraction II the one described above. The final supernatant is quite clear and yields



on high speed centrifugation (100,000 g) only a small amount of material similar to that obtained in fraction II.

It is to be noted that the general outline of the fractionation is such as would be used in the isolation of mitochondria from almost any tissue. In a following paper (12) it will indeed be shown that the isolated particles (fraction II) do have a number of enzymatic characteristics peculiar to mitochondria.

Due to the extreme technical difficulties the tough tissue poses, especially with respect to homogenization, the yield of the procedure is not very good. Only about one per cent of the initial dry weight of the aortic tissue is recovered in fraction II. If the time of blending in the high speed homogenizer is increased, the yield also increases, but this would probably produce less intact particles with respect to metabolic functions.

LIPIDE COMPOSITION OF FRACTION II

The lipids of *fraction II* were analyzed and compared with those of the intact aortic tissue. The values obtained from the analysis of a typical sample are given in Table I, together with the range of variation of individual determinations.

TABLE 1

	Fraction II			Intact Aorta
	Typical Series	Variation Limits	Number of Determinations	
<i>Total lipid</i> per cent of dry weight	18.4	17.2—21.0	11	2.6
<i>Cholesterol</i> per cent of dry weight	2	7.4— 2.1	11	0.3
per cent of total lipid	12.0	8.0—12.2	11	11.6
<i>Phospholipid</i> per cent of dry weigh	9.25	6.9— 9.5	10	1.5
per cent of total lipid	55	44.3—58.0	10	56.0
<i>Molar ratio</i> Cholesterol/phosphorus	1 : 2.3	1 : 2.8—1 : 2.2	5	1 : 2.3

The total lipid content of fraction II is quite high, about 18 per cent. The analysis shows that about 12 per cent of this lipid can be accounted for as cholesterol and about 55 per cent as phospholipids.

When these values are compared with the corresponding ones of intact aortic tissue, the considerable enrichment of lipids in fraction II comes to view. Thus the total lipid content of this fraction is 6 to 7 times higher than that of the intact tissue. However, the proportion of cholesterol and phospholipids is the same in both cases. No selective isolation of one or the other lipid class has thus taken place.

When the values for the intact aorta in Table I are compared with those of Buddecke (4), it becomes evident that only a very coarse agreement can be found. Our total lipid values are low in comparison with Buddecke's, and his values for cholesterol are higher, while those for the phospholipide are lower than ours. A species difference may be the reason for these disagreements, as Buddecke's analyses were made on human aorta. The different procedures used in the extraction of lipides may also contribute to the differences. In our experience the extraction method of Folch *et al.* using chloroform-methanol mixtures (9) is more selective in that it excludes non-lipide materials more efficiently than the alcohol-extraction procedure used by Buddecke. In some preliminary experiments, where the total lipid was determined by extraction with hot alcohol, higher values were obtained than with the chloroform-methanol-extraction.

DISCUSSION

In the study of the metabolism of a dense type of connective tissue, such as the aortic wall, the difficulties in the preparation of metabolically active fractions of the tissue poses severe limits. The authors therefore hope, that the isolation of the above described particulate fraction might be of value in this respect. Some of its metabolic properties are dealt with in an accompanying paper (12). Here only the possible implications of its high lipid content will be discussed. As was mentioned the total lipid content of the isolated fraction II is about 6—7 times higher than that of the intact aortic tissue. The composition of the lipides is in both cases the same.

Although the yield is poor, the similarity in the lipid distribution pattern could be taken to indicate, that most of the aortic lipid occurs in connection with particulate entities of the kind isolated in fraction II. Enzymatically fraction II reminds of mitochondria (12), and the question therefore arises whether the observed lipid pattern has any similarity with that known for mitochondria in general. Indeed the preponderance of phospholipids conforms with the fact that mitochondria from liver and heart muscle contain almost only phospholipids. Cholesterol is found, however, in amounts greatly exceeding those seen in liver or heart mitochondria. If the aortic lipids thus are mainly thought to reside in the mitochondria or to be closely connected with them the question of the role of the mitochondria in the alterations of the lipid pattern in arteriosclerosis immediately arises.

SUMMARY

The isolation of a homogeneous, particulate fraction from pig aortic tissue is described. The lipid content of this fraction has been studied. It is found to contain 18 per cent lipides of its dry weight, as compared to about 3 per cent in the original tissue. The distribution of this lipid between cholesterol and phosphatides is the same in the isolated fraction and intact aorta, namely about 55 per cent phospholipides and about 12 per cent cholesterol.

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OXIDATIVE METABOLISM OF A PARTICULATE FRACTION FROM PIG AORTA

by

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In the study of cellular metabolism the isolation of well-defined subcellular components with enzymatic activities has been of major importance. Certain tissues pose in this respect considerable difficulties, especially due to their extreme strength and resistance to homogenization and fractionation. One such tissue is the arterial wall. The pathological changes taking place in the arterial wall in arteriosclerosis has focussed interest on the metabolism of this tissue. It is known that a small, although definite respiration takes place in the aorta (1, 2), and that a rapid incorporation of sulfate is typical (3,5) for this tissue.

In the foregoing paper (4) the isolation of a particulate fraction from pig aorta was described. The present paper reports on some metabolic properties of this fraction. The rate of oxidation of different substrates has been studied under a number of conditions.

MATERIALS AND METHODS

The particulate fraction II was prepared as described in the foregoing paper (4). The washed fraction II obtained from about 35 pigs was suspended in isotonic sucrose to a final volume of 4–5 ml. The supernatant remaining after the separation of fraction II was used as such.

The oxygen consumption of fraction II was measured with the conventional Warburg technique. The assay mixture contained: 50 μ moles phosphate buffer, pH 7.4, 10 μ moles MgCl_2 , 5 μ moles ATP, 100 μ moles glucose, 20 μ moles glutamine, 2 μ moles UDPG, 0.007 μ moles cytochrome *c*, 3 μ moles DPN, and as substrates 20 μ moles pyruvate and 2 μ moles malate, 20 μ moles succinate, or 20 μ moles glutamate, all in the main compartment of the flask. The final volume was 2.0 ml. 0.1 ml of 6 N KOH was placed in the center well together with a filterpaper strip. When indicated one or more of the ingredients were omitted. 0.5 ml of the suspension of fraction II and 0.5 ml of the supernatant were used for assay as indicated. In some experiments, where phosphate binding was studied, 1 mg of hexokinase was added to serve as a phosphate trap. Measurements of oxygen uptake were made at either 30°C or 37°C. Readings were taken every 10 minutes after an initial equilibration period of 5 minutes. The oxygen uptake is expressed as $\mu\text{l O}_2$ per hour and mg dry weight ($= \text{Qo}_2$) of fraction II, and is based on the uptake during the first 30 minutes of the experiment.

The chemicals used were of reagent grade, and the solutions were made in glass distilled water. ATP and UDPG were purchased from Sigma Chemical Co., DPN and cytochrome *c* from Boehringer und Söhne.

RESULTS

Respiration with Various Substrates. — The respiration rates of fraction II with various substrates in absence of the supernatant appear in Table I. The highest rate is obtained with succinate. Pyruvate plus malate gives only one third of this rate and glutamate about two thirds. In absence of glucose and ATP the respiration rates were about half of the rates obtained in the presence of these substances. The increase in oxygen uptake caused by the addition of glucose and ATP was different with the different substrates, and could thus hardly be due simply to the utilization of glucose as substrate.

In Table II some results are listed showing the effect of various omissions from the reaction mixture on the oxygen uptake. The effect of the supernatant is also shown. Both with succinate and pyruvate-malate as substrates the supernatant greatly stimulates

TABLE 1

RESPIRATION OF FRACTION II IN ABSENCE OF THE SUPERNATANT
Glutamine and UDPG were omitted from the assay mixture described in the text, as was glucose except where indicated. Temperature 30°C

Substrate	QO ₂
Succinate	3.8
Succinate + glucose	6.6
Pyruvate, malate	0.8
Pyruvate, malate + glucose	2.2
Glutamate	1.8
Glutamate + glucose	4.0

TABLE 2

THE EFFECT OF CYTOCHROME C AND DPN ON THE SUPERNATANT-STIMULATED
RESPIRATION OF FRACTION II

Conditions as described in the text. Temperature 37°C

Substrate	Reactants Omitted	QO ₂	
		Cyt c and DPN Absent	Cyt c and DPN Present
Pyruvate, malate	—	4.2, 3.2	9.6, 10.4
Pyruvate, malate	supernatant	1.4, 0.8	6.0
Pyruvate, malate	glucose	3.0	5.2
Pyruvate, malate	glutamine	2.2	10.2
Pyruvate, malate	UDPG	2.2	9.4
Succinate	—	5.4	11.6, 13.8
Succinate	supernatant	3.6	7.8
Succinate	glucose, glutamine	—	8.8
Succinate	UDPG	—	10.0

the respiration rate. In addition, when cytochrome c and DPN are present, the respiration is about twofold as compared with the rate in the absence of these cofactors. The omission of glucose produces a decrease in the respiration rate similar in magnitude to that obtained when the supernatant is omitted. In the experiments with pyruvate-malate, in absence of cytochrome c and DPN, the omission of glutamine and UDPG causes a similar decrease in oxygen uptake. This was first thought to be an indication of the presence of a system synthesizing amino sugars, but as no similar effect was seen in the presence of cytochrome c and DPN, such a conclusion seems uncertain.

Respiration of Fraction II when Prepared in KCl-tris. — When fraction II was prepared, instead of in 0.25 M sucrose, in a medium containing 0.1 M KCl and buffered with 0.05 M tris-chloride buffer, pH 7.4, the respiration rates in Table III were obtained. The rates

TABLE 3
RESPIRATION OF FRACTION II PREPARED IN A KCL-TRIS-MEDIUM
Assay conditions as in Table I. No cyt c or DPN present, nor any supernatant.
Temperature 30°C

Substrate	QO ₂
Succinate	9.8
Succinate + glucose	10.8
Pyruvate, malate	1.2
Pyruvate, malate + glucose	1.2
Glutamate	1.6

with pyruvate-malate and glutamate as substrates are comparable to those obtained under similar conditions with the sucrose-fraction II, whereas the O₂ uptake rates with succinate are much higher.

Oxidative Phosphorylation. — In some experiments the uptake of inorganic phosphorus was determined. No uptake was found, and it was therefore concluded that fraction II could not catalyze oxidative phosphorylation.

DISCUSSION

The results presented above show that aortic tissue can be treated as other tissues and that fractions obtained from it show an active metabolism. The fractionation procedure used to obtain fraction II much resembles the methods used for the preparation of mitochondria. It is therefore not very surprising that this fraction shows some properties closely resembling those of mitochondria. Fraction II catalyzes the oxidation of succinate, pyruvate and glutamate. The oxidation rate is considerably increased by the addition of cytochrome c and DPN. This fact indicates that some damage has occurred to the mitochondrial system, as does the fact that no oxidative phosphorylation could be detected. It must therefore be concluded that if the particles of fraction II are to be called mitochondria, their highly damaged condition must be

kept in mind. The particulate fraction II more resembles mitochondria still containing an intact electron transport chain with some of the DPN-linked dehydrogenases attached to it, but devoid of the properties leading to oxidative phosphorylation.

When the results presented in this paper are compared with oxidation rates found for intact aortic tissue, a considerable purification of the oxidative system comes to view. For rat aorta Briggs *et al.* (1) found Qo_2 values of about 2.5 with succinate as substrate, and about 1.4 with pyruvate. The rate with glucose was about 1.1. Henderson and McDougall (2) studied the respiration of aortic tissue from several different species and arrived at similar rates as Briggs *et al.* for the rat. However, in larger animals the rate was considerably lower; in pig aorta, for instance, it was only about 0.4. Henderson and McDougall used a medium containing lactate, glutamate and fumarate as substrates, and their rates should perhaps best be compared with the oxidation rates obtained with glutamate or pyruvate in this investigation. Hilz and Utermann (3) also report Qo_2 values for the aorta of the rat, and arrive at values slightly above 2 with succinate as substrate in good agreement with the other authors mentioned. The oxidation rates obtained in this study are considerably higher. Under optimal conditions, a Qo_2 of 11–14 is obtained with succinate, and about 10 with pyruvate and malate, all in the presence of glucose. This indicates at least a 5–7-fold purification of the mechanism catalyzing the oxidation. This purification is the same as that obtained for the lipide, as explained in the foregoing paper (4). It is therefore very tempting to assume that these two properties, the lipide content and the oxidation rate are somehow interconnected. It might mean, that a major portion of the tissue lipide in the aorta occurs in the mitochondria. Such a case focusses considerable interest on the role of mitochondria and their metabolic properties in the metabolism of lipides in aortic tissue, and thus on the question of the pathogenesis of arteriosclerosis.

SUMMARY

The respiration of a particulate fraction isolated from pig aorta was studied. Under optimal conditions a Qo_2 value of about 14 was obtained with succinate as substrate, whereas pyruvate and

malate gave a value of 10. The preparation also oxidized glutamate. The rates obtained indicate a 5—7-fold purification of the oxidative system, when compared with data on the respiration rates of intact aorta in the literature.

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CLINICAL EVALUATION OF A SERUM TRYPSIN DETERMINATION METHOD IN PANCREATIC DISEASE

by

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(Received for publication September 2, 1960)

Nardi and Lees (5), in 1958, introduced a new diagnostic test for pancreatic disease, the serum trypsin or arginine exopeptidase determination. The specific substrate they utilized was α -benzoyl-L-arginine amide hydrochloride. According to the authors, this compound is hydrolyzed extremely rapidly by trypsin to yield benzoyl-L-arginine and ammonia. To their knowledge, no circulatory enzyme in blood other than trypsin will produce this hydrolysis. The rate of this reaction is proportional to the enzyme concentration. The test was done by mixing the patient's serum with the substrate for a period of one hour, and the ammonia formed was titrated by modification (2) of the Conway microdiffusion method (1).

The findings of the authors (3, 4, 5) suggested that in the presence of pancreatic disease, *e.g.* carcinoma of the pancreas, there was a significant elevation of the serum trypsin determined with this method. The serum trypsin determination proved a more sensitive and reliable index of pancreatic disease than either the serum amylase or lipase.

MATERIAL, METHOD, AND RESULTS

In order to control the clinical usefulness of the test described we have performed it in 21 patients with various pancreatic diseases and in 23 controls. The method of Nardi and

Lees (3, 4, 5) was followed in detail. According to the authors, the amount of acid used in the final titration was standardized against known concentrations of lyophilized crystalline trypsin (Tryptar, Armour), and the results were expressed as units of tryptic activity. According to the authors, 100 units were arbitrarily established as the maximum normal value. The determinations were made in triplicates.

The composition of the series and the results appear from Table 1.

TABLE 1

RESULTS OF TRYPSIN DETERMINATIONS IN CONTROLS AND IN VARIOUS PANCREATIC DISEASES

Controls:

Healthy persons:

Age Sex Trypsin units

42	f	44
25	"	72
34	"	59
61	"	83
64	"	100
56	"	72
52	m	50
41	"	93
37	"	64
28	"	79

Mean 71.6

Hospital patients without known gastrointestinal diseases:

Age Sex Trypsin units

65	f	78
62	"	69
51	"	98
61	"	52
54	m	78
63	"	50

Mean 70.8

Hospital patients with diseases which could be in connection with pancreas:

Age Sex Trypsin units

66	m	43
69	f	83
64	"	29
62	"	58
53	m	79
55	f	54
60	m	40

Mean 55.1

Diagnosis

Hepatitis interstitialis
" "
Hepatitis chron.
" "
Hepatitis subac.
St. post pancreatit. ac. Cholelithiasis
Carcinoma metast. hepatis et pulm.

*Pancreatic diseases:**Acute pancreatitis:*

Age	Sex	Trypsin units	Amylase units	
			serum	urine
63	f	84	64 → 4	1024 → 16
30	»	43	32 → 4	1024 → 24
76	»	98	256 → 32	4090 → 256
50	m	78	115 → 16	1210 → 117
48	»	106	8 → 4	32 → 16
42	»	22	256 → 32	2046 → 256

Mean 71.8

*Carcinoma of pancreas
(proved by laparotomy):*

Age	Sex	Trypsin units
68	f	60
62	»	52
66	»	90
58	m	113
41	»	97
49	»	153
63	»	53
61	»	70
89	»	84

Mean 85.7

*Chronic pancreatitis
(proved by laparotomy):*

Age	Sex	Trypsin units
53	f	110
57	»	84
49	»	93
51	m	50
42	»	60
45	»	54

Mean 75.1

As it appears from the table, there were no significant differences in the trypsin values between the patients with pancreatic disease and the controls.

SUMMARY

According to our experience with the serum trypsin determination method, introduced by Nardi and Lees, there were no significant differences in the trypsin values between the patients with pancreatic disease and the controls.

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EXPERIMENTAL ENTEROCOCCAL INFECTION IN MICE

by

TIMO KOSUNEN

(Received for publication June 30, 1960)

The number of bacteria in the organs of mice inoculated intravenously with a *Streptococcus faecalis* strain have been determined by the microbial enumeration method (2). The characteristics of the infection have been confirmed by Erlandsson, Gagliardi and Fisher (1). In the present study infections caused by seven different *Streptococcus faecalis* strains were studied by the microbial enumeration method (3, 2).

MATERIAL AND METHODS

Mice. — 210 male mice of the Swiss Albino Webster strain, weighing approximately 18 g when challenged, were used.

Enterococci. — Three out of the seven strains were isolated from urine specimens, three from eczemas and one from an infected umbilical cord stump. The bacteria grew on tellurite plate (tellurite 1/2500), resisted 30 minutes heating at 60° C and grew in broth containing 6.5 per cent of sodium chloride. They hydrolysed esculin and formed acid from glucose, lactose and mannitol.

Technique. — A 24-hour broth culture of bacteria was diluted 1:2 and 0.2 ml of the dilution was inoculated intravenously into one of the lateral tail veins of the mice. The viable counts ranged from 6×10^7 to 9.2×10^7 bacteria/inoculum.

Aided by a grant from the Oscar Öflund Foundation.

At appropriate intervals five mice from each group were killed, the liver, spleen and kidneys removed, and the number of culturable *Streptococcus faecalis* organisms in these organs determined by the microbial enumeration method (3, 2).

RESULTS

No deaths occurred during the experiments.

Kidneys. — The number of culturable enterococci obtained from the kidneys of the mice are given in Table 1. On the first post-

TABLE 1

THE MEANS OF THE LOGARITHMS OF THE NUMBER OF ENTEROCOCCI PER ML OF KIDNEY IN MICE. THE VALUES ARE MEANS OF FIVE ANIMALS EXCEPT IN THE CASE OF THE STRAIN K, WHERE THEY ARE MEANS OF 15—40 ANIMALS, TAKEN FROM AN EARLIER STUDY (2)

Day	Bacterial Strain							
	A	B	C	D	E	F	G	K
1	4.82	4.67	4.67	4.59	4.77	4.60	4.74	4.52
3	6.41	4.98	5.39	4.66	6.15	5.51	5.59	5.95
5	6.67	7.24	6.82	6.23	5.84	7.07	6.02	6.56
7	5.36	6.48	5.40	6.32	6.62	5.70	6.11	7.11
9	6.86	5.00	4.95	4.40	7.27	6.25	5.71	6.92
12	6.53	5.60	4.21	5.09	6.14	5.36	4.95	6.48

challenge day the logarithms of the numbers of bacteria were within 0.3 logarithm unit. In all of the strains studied the bacterial population increased during the first five post-challenge days, but

TABLE 2

THE MEANS OF THE LOGARITHMS OF THE NUMBER OF ENTEROCOCCI PER ML OF LIVER IN MICE. THE VALUES ARE MEANS OF FIVE ANIMALS EXCEPT IN THE CASE OF THE STRAIN K, WHERE THEY ARE MEANS OF 7—34 ANIMALS, TAKEN FROM AN EARLIER STUDY (2)

Day	Bacterial Strain							
	A	B	C	D	E	F	G	K
1	6.95	6.92	5.92	6.52	6.19	6.01	6.13	5.77
3	5.42	5.60	5.38	5.49	5.30	4.68	4.37	4.85
5	4.70	3.89	5.69	4.48	3.99	3.90	5.17	3.89
7	2.50	3.59	4.98	4.44	4.58	2.73	3.64	2.83
9	3.08	2.73	3.56	3.68	3.40	2.65	3.74	1.77
12	1.86	2.83	3.45	1.60	3.46	1.31	2.94	1.90

TABLE 3

THE MEANS OF THE LOGARITHMS OF THE NUMBER OF ENTEROCOCCI PER ML OF SPLEEN IN MICE. THE VALUES ARE MEANS OF FIVE ANIMALS EXCEPT IN THE CASE OF THE STRAIN K, WHERE THEY ARE MEANS OF 7-33 ANIMALS, TAKEN FROM AN EARLIER STUDY (2)

Day	Bacterial Strains							
	A	B	C	D	E	F	G	K
1	5.85	5.77	5.88	5.91	5.68	5.63	5.63	4.89
3	4.69	4.91	4.23	4.19	4.78	3.89	4.39	3.37
5	4.04	1.67	4.25	3.77	3.68	2.49	4.05	1.52
7	1.93	2.24	3.67	2.98	2.35	2.27	1.55	1.79
9	2.07	1.03	1.61	0.79	2.86	1.44	2.82	0.62
12	1.86	0.67	1.65	0	1.59	0.44	1.27	0

after the seventh day a decreasing trend was seen. The daily values were in general similar, ranging from 4.21 to 7.27 and showing no significant differences between the infections caused by various strains. Macroscopical abscesses were found in the kidneys from the third post-challenge day on.

Liver and spleen. — The results of the bacterial enumerations performed on the livers are given in Table 2, and of those performed on the spleens in Table 3. On the first post-challenge day the logarithms of the numbers of bacteria in the liver were within one logarithm unit, and in the spleen within 0.3 logarithm unit, thus showing homogeneity of the infections at the beginning of the experiment, as did the values obtained from the kidneys.

DISCUSSION

It seems to be the rule that somewhat larger bacterial populations are met with in the liver than in the spleen, and that the infection also lasts longer in the former. In both organs, however, a decreasing trend in bacterial counts was evident, and no macroscopical lesions were observed.

The infection was most persistent in the kidneys, where the bacterial population increased initially after the challenge. The highest values in the kidneys, obtained on the fifth to ninth post-challenge days, were followed by a decrease towards the end of the observation period. The number of bacteria found in the kidneys,

spleen and liver roughly corresponded to the results obtained earlier when a slightly smaller inoculation dose was used (2).

The relatively low virulence of enterococci intravenously inoculated into white mice (1, 2) was confirmed in this study, in which no deaths due to the infection occurred. The enumeration of the enterococci from the organs did not reveal any striking differences in virulence between the *Streptococcus faecalis* strains. The persistence and regular presence of the enterococci in the kidneys was a conspicuous feature and this model seems to be useful for studies on experimental enterococcal pyelonephritis. According to Erlandsson, Gagliardi and Fisher (1) the applicability of the model is not restricted to acute infections — these workers followed the process in the kidneys up to five months.

SUMMARY

Experimental infections were induced in mice with seven enterococcal strains, which were inoculated by the intravenous route. The number of bacteria in the kidneys, spleen and liver was determined during a 12-day observation period. In the kidneys the enterococci, after an initial increase, showed a decreasing trend during the last days of the experiment. In the liver and spleen the bacterial population decreased from the first post-challenge day on. Bacterial enumeration of this non-lethal infection revealed no significant differences in virulence between the *Streptococcus faecalis* strains studied.

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EXPERIMENTAL MONILIAL INFECTION IN MICE

VIRULENCE STUDIES

by

TIMO KOSUNEN

(Received for publication August 31, 1960)

Intravenous inoculation of *Candida albicans* organisms into mice results in a fatal infection the brain, heart, spleen and kidneys being the most regularly affected organs (1). Since the survival rate does not give an adequate picture of the virulence of the microbes in short-term experiments (8, 9), the individual organs have also been subjected to microbial enumerations (4). In the present study, severity of the infections caused by seven *Candida albicans* strains has been evaluated by making microbial enumerations from the kidneys of experimentally infected mice.

MATERIAL AND METHODS

Mice. — 210 male mice of the Swiss Albino Webster strain were used. At inoculation their weight ranged from 18 to 22 grams.

Candida Albicans Strains. — The microbes were isolated from clinical specimens, two strains from sputum (D, E), one from a pharyngeal swab (A), two from lesions of the finger nails (B, G), one from urine (F) and one from lochia putrida (C). They formed grayish-white colonies on blood agar with a distinct «yeasty» odor, formed acid and gas from glucose and acid from sucrose, but did not split lactose.

Technique. — 24-hour broth cultures of *Candida albicans* strains were diluted with saline and 0.2 ml of the dilution was injected intravenously into one of the lateral tail veins of the mice. 30 mice were inoculated with a strain; the viable counts of the inocula ranged from 2.4×10^3 to 3.4×10^3 cells. The number of culturable *Candida albicans* organisms in the kidneys of five mice from each group was determined at appropriate intervals by the microbial enumeration method (4, 7).

RESULTS

The means of the logarithms of the number of culturable *Candida albicans* organisms in the kidneys of the mice are given in Table 1. With all strains the first enumerations, performed one day after challenge, gave logarithmic values between 3 and 4. From the third day on, macroscopical abscesses were seen in the kidneys. Strains A, C, D, E and F caused infections in which the microbes increased rather similarly during the observation time. With strain B the population showed a tendency to remain slightly smaller than with the other strains, whereas with strain G the highest numbers of yeast cells were seen after the first post-challenge day. This strain also caused the greatest number of deaths, seven; with strain A the number was two, with B, C and F one and with D and E nil. As a whole, the microbial counts in infections with the various strains did not differ significantly.

TABLE 1

THE MEANS OF THE LOGARITHMS OF THE NUMBERS OF CULTURABLE *CANDIDA ALBICANS* CELLS PER ML OF KIDNEY IN MICE. THE VALUES ARE MEANS FOR FIVE MICE EXCEPT IN THE CASE OF THE STRAIN G ON THE TENTH DAY, WHERE THE VALUE IS THE MEAN FOR THREE MICE

Day	Microbial Strain						
	A	B	C	D	E	F	G
1	3.89	3.26	3.47	3.05	3.54	3.48	3.88
3	3.31	3.42	5.00	4.66	4.26	4.05	5.04
5	4.76	2.84	4.32	4.79	4.54	3.66	6.20
7	5.51	2.89	4.04	5.91	4.71	5.04	6.62
10	6.03	4.70	5.99	6.47	4.74	5.04	6.68

DISCUSSION

Among *Candida* species *Candida albicans* has been shown to be the most virulent to mice (5). Apart from some *Candida stellatoidea* strains, *C. albicans* strains are the only ones that form filaments in the tissues (3). This has been considered to be a factor in the virulence, since the filaments are phagocytized with difficulty (9). In mice inoculated with an old laboratory strain of *C. albicans* in doses closely corresponding to those used in the present study, microbial enumeration revealed increasing numbers of yeast cells in the kidneys (4). In the present study, in agreement with earlier investigations (1, 2, 4, 5, 6), cells of the seven other *C. albicans* strains used accumulated in the kidneys of mice inoculated by the intravenous route. The course of the infections, as evaluated by making counts of yeast cells from the kidneys, was much the same as when the old laboratory strain was used (4).

In the present study the microbial counts from the kidneys did not differ significantly with the various *C. albicans* strains. The death rates, ranging from nil to seven, suggested differences in virulence between the strains, but the results of the microbial enumerations failed to give further light on the matter.

SUMMARY

The virulence of seven *Candida albicans* strains was studied in mice inoculated by the intravenous route. 30 mice were challenged with each strain. The death rates ranged from nil to seven with the various strains. The number of yeast cells in the kidneys was determined by the microbial enumeration method over a period of ten days. With all strains the microbial populations showed a tendency to increase but there were no significant differences in microbial counts between the strains.

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DETERMINATION OF THE COAGULASE PRODUCTION OF STAPHYLOCOCCUS AUREUS IN CAPILLARY TUBES

by

TIMO KOSUNEN

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In 1959, Griffith and Ostrander published a method for detecting the coagulase production of staphylococci in capillary tubes (1). In the present study minor modifications of this capillary tube method are described, and the reactions are compared with those obtained with the usual tube method.

MATERIALS AND METHODS

Staphylococci. — 171 strains isolated from clinical specimens were studied.

Coagulase Reactions. — A. The usual method, in which a loopful of bacteria from a plate culture on blood agar was transferred to a tube containing 1 ml of 1 : 10 dilution of human plasma in saline.

B. The capillary tube method devised by Griffith and Ostrander (1), in which a layer of 18-hour broth culture about 1 inch deep, followed by a layer of undiluted plasma about 1½ inches deep, was allowed to enter the tube.

C. A capillary tube method like B above, but the plasma was allowed to enter the capillary tube first.

D. A capillary tube method in which a loopful of staphylococci from a plate culture was suspended in broth. The suspension was allowed to enter the capillary tube under the plasma layer.

E. A capillary tube method in which the capillary tube containing a 1-inch layer of plasma was pressed vertically through a

bacterial colony and the medium beneath it, which thus formed a plug in the lower end of the tube.

In the capillary tube methods the tubes were held in a vertical position with plasticine. All tubes were incubated for 18 hours at 37° C. In positive reactions a distinct clot was formed either at the interface of the media or throughout the fluid.

RESULTS

Irrespective of the method used the 18-hour readings of the reactions, given in Table 1, were in complete agreement. Rough estimation of the intensity of the reactions revealed that the positive

TABLE 1

NUMBER OF COAGULASE POSITIVE AND NEGATIVE STAPHYLOCOCCAL STRAINS AS DETERMINED BY DIFFERENT TECHNIQUES AFTER 18 HOURS' INCUBATION AT 37° C

Method	No. of Strains Studied	Positive	Negative
Usual tube	171	166	5
Broth culture-plasma	171	166	5
Plasma-broth culture	171	166	5
Plasma-broth suspension	171	166	5
Plasma-plate colony	171	166	5

reactions were weakest in those capillary tubes in which the broth suspensions or plate colonies of staphylococci were under the plasma layer. When the tubes had been incubated for three hours, only indistinct clot formation was found, and the reactions varied according to the method used. After six hours' incubation stronger positive reactions were seen, and the results obtained with different methods were in general in agreement. A further 12 hours' incubation was needed, however, for the reactions to become intense and distinct with all the methods used. Weak and indistinct reactions were obtained in attempts to use 1:5 diluted plasma in capillary tube tests.

DISCUSSION

The preliminary distribution of the plasma in drops, necessary in using the method of Griffith and Ostrander (1), can be omitted by allowing the reactants to enter the capillary tube in reverse order. In this way a little labour and materie is saved, since the plasma can be taken with the capillary tube direct from the vial.

In the capillary tube method colonies grown on agar plates can also be used directly by pressing the tube through the colony and the agar medium beneath it. This modification of the reaction requires a prolonged incubation. After 18 hours' incubation the reactions were unmistakeable.

Qualitatively, the results were the same with the capillary tube method whether a staphylococcal suspension or a broth culture was used under the plasma layer. As regards intensity, however, the reaction obtained with a suspension of staphylococci was inferior to that obtained with a broth culture. In the performance of coagulase test, the capillary tube methods save labour and material. Their main advantage, however, is that they allow cheap and easy disposal of used infectious material and so obviate post-usage handling of tubes, with the sterilizing and washing procedures involved (1).

SUMMARY

Slight modifications to the capillary tube method for studying the coagulase reaction of staphylococci (1) are described. Besides broth culture, broth suspension and plate culture could be used as the starting material. The results obtained with the various capillary tube methods were in full agreement mutually as well as with the results obtained by the usual tube method.

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INFLUENCE OF THYROTROPIC HORMONE ON THE LEUCINE AMINOPEPTIDASE ACTIVITY IN THE THYROID GLAND OF THE RAT

by

VÄINÖ K. HOPSU and S. TALANTI

(Received for publication September 14, 1960)

In previous studies (7) the writers showed that in the thyroid gland of the rat a decrease in histochemically demonstrable activity of leucine aminopeptidase was produced by the administration of methyl-thiouracil. The mechanism through which this effect of methyl-thiouracil operates is obscure. It has been found that thiouracil blocks the synthesis of thyroxine (*e.g.* 4) because the accumulated iodide ion can no longer be used for the iodination of tyrosine. Since the concentration of the thyroid hormone in the blood stream falls, signs and symptoms of hypothyroidism develop. In an effort to maintain the normal rate of secretion of the thyroid hormone, the anterior pituitary gland secretes abnormally large quantities of thyrotrophic hormone. The present experiment, in which histochemically demonstrable leucine aminopeptidase activity in the thyroid gland of the normal rat was compared with that of the rat treated with thyrotrophic hormone, was carried out with an intention to elucidate the mechanism through which methyl-thiouracil acts upon the activity of this enzyme in the thyroid gland.

MATERIAL AND METHODS

Twenty adult male albino rats, weighing about 230 g, were used. They were allowed the same diet and drinking water *ad libitum*, and they were housed in a room with constant temperature. Ten

rats were given 15 IU of thyrotrophic hormone («Ambinon», Organon) subcutaneously daily for 4 days. The control animals received similar volumes of saline. Two days after the last injection the rats were sacrificed by rapid decapitation without anaesthesia.

The thyroid glands were removed immediately after sacrificing and frozen with dry ice. They were cut with a Pearse cold microtome in 5 to 30 μ thick sections. Since the sections of the test animals had to be exactly as thick as those of the corresponding controls, the thyroids of the two types of animals were always sectioned in couples simultaneously, and placed on the same slide. The demonstration of leucine aminopeptidase was carried out by the method of Nachlas *et al.* (5). Incubation time was 15–120 minutes.

For the histological determination of the effect of thyrotrophic hormone, a small piece of each thyroid gland was taken, fixed in 10% formalin and embedded in paraffin. The sections were stained with haematoxylin-eosin.

RESULTS

The typical effect of thyrotrophic hormone on the thyroid gland was encountered in sections stained with haematoxylin-eosin.

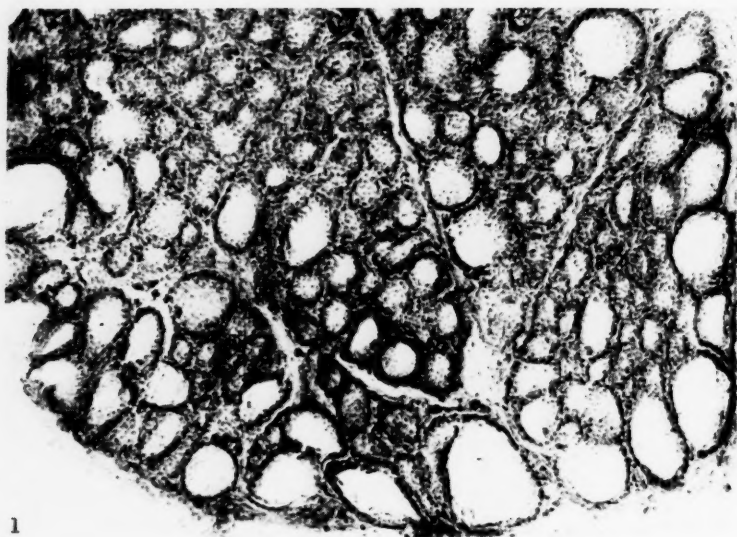


Fig. 1. — Leucine aminopeptidase activity in the thyroid gland of the control rat. Incubation time 30 min. 120 \times .

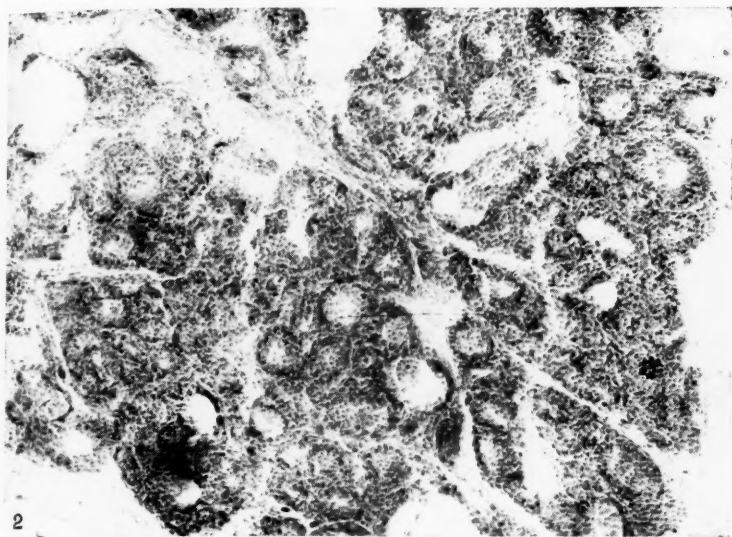


Fig. 2. — Depressed activity of leucine aminopeptidase in the thyroid gland of a rat treated with thyrotrophic hormone. Incubation time 30 min. 120 \times .

A moderate activity of leucine aminopeptidase occurred in the thyroid glands of the control rats. It was most clearly found in the epithelium cells of the follicles. Slight activity was also present in the colloid. Interfollicular connective tissue was negative. The mast cells showed, however, a clear response. The parathyroid glands displayed an intense activity (Fig. 1).

Activity of the thyroid epithelium of the test animals was practically absent (Fig. 2), whereas activity of the mast cells and that of the parathyroid glands was the same as that of the control animals.

DISCUSSION

The result shows that the effect of thyrotrophic hormone on histochemically demonstrable activity of leucine aminopeptidase in the thyroid gland of the rat is similar to that of methyl-thiouracil. It appears likely that thiouracil acts not directly on the activity of this enzyme in the thyroid gland but the action is mediated by the anterior pituitary gland. Other eventual mechanism may, however, exist too.

The functional significance of leucine aminopeptidase in tissues is not very well understood. Apparently it is associated with the protein metabolism. According to Pearse and Tremblay (6) leucine aminopeptidase plays a part in the protein synthesis of the parathyroid glands. It may be obviously also connected with the proteolytic processes. No conclusive evidence of possible protein synthesis in the thyroid gland has been observed, while the proteolytic enzymatical system of the thyroid gland has been found (1, 2, 3, 8). Yet, the significance and the structure of this system is still unresolved. It is possible that it concerns with the hydrolysis of colloid protein and with the eventual absorption of the physiologically active products. This system might be of complex nature. Gersch and Baker (1) found that the administration of thyrotrophic hormone to rats causes a decrease in the concentration of total protein of colloid which can be correlated with an increase in proteolytic activity. To contrast for this finding Loughlin *et al.* (3) observed that the administration of thyrotrophic hormone and that of methyl-thiouracil produces a decrease in the concentration of «cysteinyl-tyrosinase» as well as in that of proteinase. It is apparent that thyrotrophic hormone and methyl-thiouracil exert the same effect on leucine aminopeptidase as on the components of the proteolytic system of the thyroid gland stated in the above-mentioned investigation. In histochemical investigations, as a matter of fact, the concentration of enzyme is measured while the amount of activity is estimated by a visual test on the base of intensity in staining. Actually, one can put forward the assumption that leucine aminopeptidase is part of the proteolytic system of the thyroid gland.

SUMMARY

In rats treated with the thyrotrophic hormone a marked decrease in the amount of leucine aminopeptidase of the thyroid gland as compared with that of the control animals could be histochemically demonstrated. The relationship of the present finding to the decrease in activity of leucine aminopeptidase following methyl-thiouracil administration previously found in the thyroid tissue is elucidated. A eventual role of leucine aminopeptidase in the proteolytic system of the thyroid gland is also discussed.

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EFFECTS OF THIORACIL FEEDING ON THE ADRENAL MEDULLA OF THE RAT

by

VÄINÖ K. HOPSU

(Received for publication September 21, 1960)

Several studies have shown that thiouracil feeding causes changes in the adrenal medulla. Marine and Bauman (15) reported pronounced medullary hypertrophy and hyperplasia in the adrenal medulla of the rat after 2—4 months treatment with thiouracil. Adams and Bull (1) and Adams and Buss (2) reported adrenal medullary hypertrophy after injections of thiourea into developing chicken eggs.

In an earlier study by the present writer no marked changes were observed in the total volume of the adrenal medulla of the mouse after treatment with thiouracil for several months. The relative volumes of the two disjunct medullary tissues were, however, changed: the volume of the adrenaline-containing parenchyma was decreased, while the volume of the noradrenaline-containing parenchyma was markedly increased and its noradrenaline content was elevated (11).

In the above-mentioned early studies on the rat and the chicken (Marine and Baumann, 15; Adams and Bull, 1; Adams and Buss, 2) no differentiation was made between the two types of medullary tissue, as might be expected because the histochemical methods for selective demonstration of noradrenaline were introduced later. Therefore it seemed to be worth while to reinvestigate the effects of thiourea on the rat adrenal medulla using histochemical methods for demonstrating the two types of medullary tissue. Such a study is reported in the present paper.

MATERIAL AND METHODS

Fourteen rats were used. To 7 rats 0.3% methyl thiouracil was included in the ordinary laboratory diet for a period of five weeks. The administration of thiouracil was stopped 8 days before the decapitation of the animals. Both adrenals of every rat were removed for investigation.

Iodate Reaction. — The left adrenals were treated for 24 hours in a saturated solution of potassium iodate for the iodate reaction (10). They were then fixed in 3.5% formaldehyde for another 24 hours and sectioned serially at 50 μ with a freezing microtome. The total medullary area and the area covered by iodate-positive medullary cells were planimetrically measured from the whole series of sections and the total volume of the medulla and the relative volume of the iodate-positive or noradrenaline-containing medullary tissue were calculated from these values (7).

Chromaffin Reaction. — The right adrenals were fixed in a dichromate-formalin mixture and the medullary volume was measured from a complete series of sections.

Statistical Analysis. — To compare the significance of the differences of the means, Student's *t*-test was used.

RESULTS

The weights of the experimental animals, and of their adrenal glands, the medullary volumes and the volumes and percentages of the iodate-positive, *i.e.* noradrenaline-containing, cells in the adrenal medulla of the thiouracil-fed and the control animals are listed in Table 1. It will be seen that the loss in body weight of the thiouracil-fed rats was significant and that the adrenal weight was also significantly lower than that of the controls. The relative adrenal weight was, however, approximately the same in both groups. The total volume of the adrenal medulla of the thiouracil-treated rats was approximately the same as that of the controls the difference being not statistically significant. The volume of the iodate-positive tissue was, however, doubled during the thiouracil feeding as compared with the control animals, a change which was statistically significant, $P < 0.01$. The proportion of the nor-

TABLE 1.

FINAL BODY WEIGHT, WEIGHT OF THE ADRENAL GLANDS, TOTAL VOLUME OF THE MEDULLA AND ABSOLUTE AND RELATIVE VOLUMES OF THE IODATE-POSITIVE TISSUE OF THE MEDULLA AFTER THIOURACIL FEEDING

Treatment	No. of Animals	Animal Weight (gm)		Adrenal Weight (mg)		Medullary Volume (mm ³)		Volume of Iodate-positive Tissue (mm ³)		Iodate-positive Tissue Volume %	
		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Thiouracil feeding ..	7	147	9.8 ²	13.2	1.5 ¹	1.38	0.16	0.32	0.08 ¹	23.2	1.87 ²
Controls ..	7	198	12.4	16.0	1.7	1.60	0.22	0.16	0.10	10.0	1.57

¹ Difference from the mean control value is significant (P 0.01).

² " " " " " " " " " " " " (P 0.001).

adrenaline-containing tissue in the whole medulla was also accordingly increased from 10% to 23%, the change being again significant (P < 0.001).

The intensity of the iodate reaction, known to indicate the concentration of the noradrenaline in the tissue (8), was clearly increased in the adrenal medulla of thiouracil-treated animals,

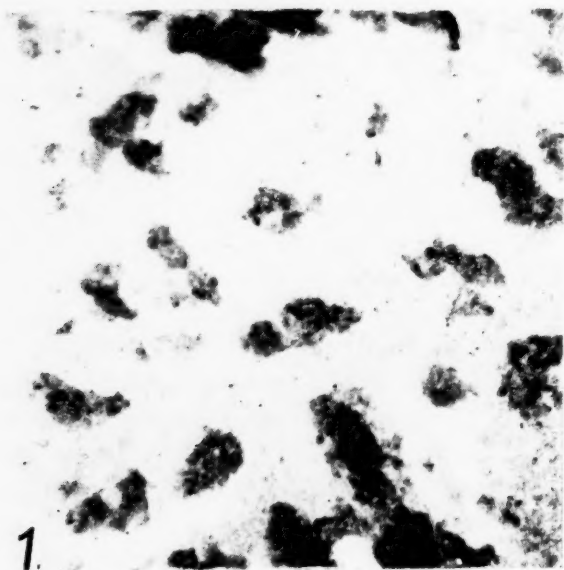


Fig. 1. — Adrenal medulla of a normal rat. Iodate reaction. $\times 90$.

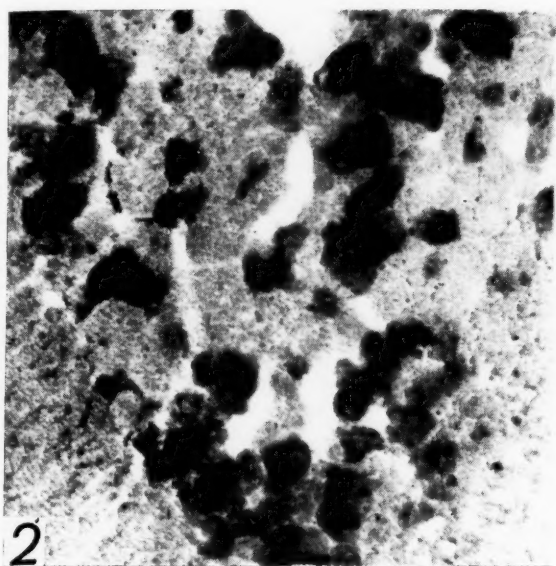


Fig. 2. — Adrenal medulla of a rat fed with methyl thiouracil. Iodate reaction. Both the amount of the iodate-positive tissue as well as the intensity of the iodate reaction are increased. $\times 90$.

especially in the iodate-positive cell islets but also in the whole background of the medulla (Fig. 1 and 2). The same change in the intensity of the chromaffin reaction was observed in all the adrenals studied.

DISCUSSION

The observations made are in very good agreement with earlier ones made on the mouse, *i.e.* the total medullary volume was not markedly affected by thiouracil, while the noradrenaline-containing tissue showed a marked increase in its volume either through hypertrophy or hyperplasia of the medullary cells. The increased intensity of the iodate reaction as well as that of the chromaffin reaction show that the noradrenaline concentration was increased. The increase in the intensity of the chromaffin reaction over the whole medulla shows that the total catecholamine concentration was increased. In the mouse adrenals such changes were observed, however, first after treatment 4–6 months (Hopsu, 11).

The observations are in good agreement also with those by

Hököfelt (13). He found that, in the adrenal medulla of the rat, both the adrenaline and noradrenaline increased after treatment for 25 days with thiouracil, the content of noradrenaline being increased much more than that of the adrenaline. Marine and Baumann (15) also made amine determinations from the adrenal medulla of the rat after thiouracil feeding and found adrenaline to be increased; the study was, however, made at a time when very little was yet known about noradrenaline and these investigators did not differentiate between the two amines.

The nature of the mechanism which caused the hypertrophy of the iodate-positive tissue is not clear. It might be due to an intense secretory stimulation resulting in functional hypertrophy. The increased noradrenaline concentration in the background of the medulla, which normally contains almost solely adrenaline, might depend on an increased amine synthesis in these cells, for it is known that during intense amine resynthesis in the medulla, the noradrenaline concentration tends to increase above the normal level (Butterworth and Mann, 3; Callingham and Mann, 4; Coupland, 5).

Reports by Friedenswald and Buschke (9) and Di Palma and Dreyer (6) indicate that thiourea is antagonistic to adrenaline in its biological effects. Kingsbury *et al.* (14) suggest that the adrenal medullary hypertrophy obtained is caused by an increased secretion of adrenaline, necessary to compensate for the effect of thiourea. No direct evidence of increased amine secretion after thiourea is presented, however. Hököfelt (13) found no increase in the adrenaline content of the liver and heart after thiouracil feeding and an increased noradrenaline content was found only in the heart muscle.

Another possible explanation for the increased noradrenaline concentration in the adrenaline secreting cells might be a decreased rate of methylation of noradrenaline to adrenaline, the rate of other steps of the amine synthesis being normal. The observations of Hutcheon and Parker (12) suggest, however, that methylation is not affected by thiouracil. This possibility also should not solve the hypertrophy of the real noradrenaline containing tissue.

In the light of these observations it is obvious that the mechanism responsible of findings made requires further study.

SUMMARY

Albino rats were fed for 5 weeks with a diet containing 0.3% of methyl thiouracil. They were killed 8 days later.

The weights of the experimental animals and of their adrenal glands were significantly lower than those of the controls. The total medullary volume was not affected. The volume of the noradrenaline-containing or iodate-positive tissue was, however, doubled and the percentage of the iodate-positive tissue from the whole medulla was still more increased.

Intensities of the iodate as well as chromaffin reactions increased both in the specific cell islets as well as in the background of the medulla, indicating an increase in the medullary concentrations of both noradrenaline and adrenaline.

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EFFECTS OF WHOLE-BODY X-IRRADIATION ON THE NEUROSECRETORY GANGLION CELLS IN THE SUPRA- OPTIC NUCLEUS OF THE RAT¹

by

VÄINÖ K. HOPSU, V. VILJANEN, and S. TALANTI]

(Received for publication September 26, 1960)

In an earlier study by Hopsu, Talanti and Voutilainen (4) it was observed that aldehyde-fuchsin positive neurosecretory material is decreased after whole-body x-irradiation in the neurohypophysis of the rat. Decrease in the hypophyseal neurosecretory material may be dependent either on a decreased synthesis of it in the supra-optic nucleus or on an increased release of it into circulation from the neurohypophysis. Supposing that the neurosecretory material is related to the regulatory mechanism of the water metabolism, the first-mentioned possibility is in agreement with the observation of Pentz and Hasterlik (6) according to which an increase occurs in the amount of diuresis after a few days following x-irradiation.

This paper is concerning with the mechanism actually resulting to decrease in the neurohypophyseal »neurosecretory substance». The nuclear size of the neurosecretory ganglion cells in the supra-optic nucleus of x-irradiated and normal rats are measured, in order to find possible changes in the activity of the ganglion cells, which could underlie the observation made about neurosecretory material in the neurohypophysis (4).

MATERIAL AND METHODS

The series consisted of 15 rats. Eight belonged to the test group and seven served as controls.

¹ Aided by a grant from the Sigrid Jusélius Foundation.

The group of test animals was exposed to whole-body x-irradiation, the dose being 500 r. The factors were following: 180 kV, filtration 0.5 Cu, 60 cm source skin distance, 15 mA and H.V.L. 1.05 Cu. After six days the animals were decapitated without anaesthesia. The control animals were held the same external circumference as the test animals and the both groups received the same diet and water *ad libitum*. The controls were also sacrificed by decapitation at the same time as those of the test group.

The brains of all rats were fixed in 10% formalin, embedded in paraffin and sectioned at 7 micra. The sections were stained with Weigert's haematoxylin.

Nuclear diameters were determined from camera lucida drawings, magnified 2500 \times . The largest and shortest diameters of nuclei were measured from 100 to 200 cell nuclei of each animal and their results are given.

The Mann-Whitney U test (7) was used for the statistical treatment of the results.

RESULTS

The numerical values obtained are presented in table 1.

When the mean values of the nuclear areas in both groups are tested against each other using the Mann-Whitney U test, a conclusion that the nuclear sizes in these groups are not equal, is

TABLE 1
NUCLEAR AREA (= THE GREATEST DIAMETER \times THE SMALLEST DIAMETER) OF THE GANGLION CELLS IN THE SECTIONS FROM THE SUPRAOPTIC NUCLEUS OF THE X-IRRADIATED AND CONTROL RATS. MEAN \pm STANDARD ERROR (ϵ)

No.	Irradiated (cm^2) Mean $\pm \epsilon$	Control (cm^2) Mean $\pm \epsilon$
1	2.62 \pm 0.04	2.89 \pm 0.07
2	2.52 \pm 0.04	2.80 \pm 0.03
3	2.47 \pm 0.03	2.79 \pm 0.07
4	2.45 \pm 0.05	2.78 \pm 0.05
5	2.38 \pm 0.03	2.76 \pm 0.05
6	2.36 \pm 0.04	2.68 \pm 0.04
7	2.21 \pm 0.03	2.58 \pm 0.04
8	2.08 \pm 0.06	—

justified at the level $P < 0.001$, the nuclei in the irradiated animals so being clearly significantly smaller than those in the control animals. This kind of testing, indeed, disregards the deviations in the nuclear sizes of single animals, the result being, however, so clear that this remark does not cause any doubt about the correctness of the conclusion made.

DISCUSSION

It is obvious that whole-body x-irradiation causes a decrease in the volume of the nuclei in the supraoptic ganglion cells. According to the present concept the nuclear volume of a cell is a good indicator about the cell activity, wherefore a conclusion can be drawn that the secretory activity of the ganglion cells of the supraoptic nucleus is decreased after x-irradiation.

In the light of the present finding the earlier observation made by Hopsu, Talanti and Voutilainen (4) which showed that the neurosecretory material was decreased in the neurohypophysis of the rat after x-irradiation, can be declared to base on a diminished synthesis and transport of neurosecretory material from the supraoptic ganglion cells to the neurohypophysis.

Because of decreased synthesis of neurosecretory material the amount of it liberated into circulation is obviously decreased, too. Presuming that the concept of the location of the antidiuretic principle in the neurosecretory material is correct, the present finding is in good agreement with the observation of Pentz and Hasterlik (6) in which a great increase in the diuresis was observed after x-irradiation. Indeed, a number of investigators *e.g.* Hild and Zetler (3) have put forward convincing evidence in favour of this result.

It is less well known which causes the decrease in the activity of the ganglion cells in the supraoptic nucleus. It is, however, formerly confirmed that the hypothalamic nuclei are susceptible to x-irradiation; x-irradiation causes a damage in these nuclei even in doses, which do not affect the surrounding brain tissue (Arnold, 1). In fact, it appears likely that the decrease in the activity of the ganglion cells of the supraoptic nuclei is produced by a direct effect of irradiation on these cells. It is hardly probable that it is due to general stress, because it is previously observed

that after various stresses an increase takes place in the volume of the ganglion cells of the neurosecretory hypothalamic nuclei (Kivalo and Rinne, 5; Cullingham, 2).

SUMMARY

Fifteen rats were used in experiments. Eight of them were x-irradiated with 500 r six days before decapitation. The brains of the animals were fixed in formalin, sectioned and stained with Weigert's haematoxylin.

Nuclear diameters of the ganglion cells of the supraoptic nucleus were measured from camera lucida drawings in order to estimate the nuclear size. The nuclear size was significantly smaller in x-irradiated animals than in controls. The relation between this observation and earlier related findings is discussed.

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EFFECT OF 4-DEOXYPYRIDOXINE ON TRYPTOPHAN METABOLISM IN *ESCHERICHIA COLI*¹

by

J. JÄNNES and N.-E. SARIS

(Received for publication October 13, 1960)

Wood, Gunsalus and Umbreit (6) and Happold (3) have clarified the mechanism of tryptophan cleavage to indole in cells of *e. coli*. They also found that pyridoxal phosphate acts as coenzyme of tryptophanase, the specific enzyme catalysing this reaction.

Umbreit and Waddel (7) clarified the mechanism of action of the pyridoxine antagonist, 4-deoxypyridoxine (DOP), and they could demonstrate an inhibition of the activity of some enzymes dependent on pyridoxal phosphate.

Dietrich and Borries (1) found that DOP inhibits the activity of glutamic-oxaloacetic transaminase but not that of cystine desulfurase. It is evident that DOP inhibits to a very variable degree the activity of enzymes dependent on pyridoxal phosphate. The authors have now studied the effect of this antivitamin on the tryptophanase activity in whole cells of *escherichia coli*. Some effects of DOP on the oxidation of tryptophan will also be reported.

The aerobic pathway of tryptophan oxidation involves also pyridoxal phosphate-dependent enzymatic reactions. As stated by Wiss and Fuchs (5) pyridoxal phosphate is the coenzyme of kynureninase, which splits kynurenine into anthranilic acid and alanine. McCullogh (4) stated that the decarboxylation of anthra-

¹ This study has been aided by grants from Nordisk Insulinfond and Sigrid Juselius Stiftelse.

nilic acid also needs pyridoxal phosphate in the metabolism of *E. COLI*.

Because of lack of newer data concerning the effect of 4-deoxypyridoxine on tryptophan metabolism in *escherichia coli* the authors carried out the following tests.

METHODS

Cultivation of E. Coli. — The writers used throughout this study a «wild» strain of *e. coli* which was kept on plant cultures and a new inculum was made every second week. For the experiments *e. coli* was cultivated in nutrient broth with aeration. The cells were harvested and washed with physiological saline solution.

Experimental Conditions. — The tests were carried out in both aerobic and anaerobic conditions. In the aerobic tests a suspension of washed *e. coli* cells (fresh weight 60 mg/ml) in $7.5 \cdot 10^{-2}$ M tryptophan solution was continuously shaken in polyvinylacetate bottles at 25°C. The pH of the suspensions was adjusted to 7.0 in the beginning of the experiments.

Anaerobic Tests. — These were carried out in corked test tubes containing bacterial suspension (fresh weight 80 mg/ml) in 5.10^{-2} M tryptophan solution. Indole was estimated in the supernatants after 16 hours' incubation.

Chromatographic Technique. — Samples of the culture media in aerobic tests taken at different intervals and standards were applied on a circular paper and chromatograms developed with isopropanol:ammonia:water (200:10:20)

Estimation of Indole. — Indole was estimated by the method described by Gunsalus, Galeener and Steamer (2) using p-dimethylaminobenzaldehyde as reagent.

RESULTS

Aerobic Tests. — The photograph (fig. 1) taken in ultraviolet light shows the migration of fluorescent tryptophan derivatives formed during the incubation. The samples have been applied in 8 areas close to the center of the circular paper.

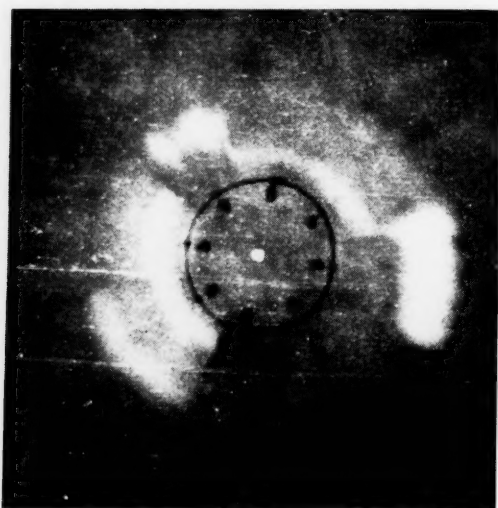


Fig. 1. — Chromatogram of fluorescent substances in the culture media of *E. COLI* under aerobic conditions.

	Incubation Time in hours	DOP Conc. M/L
Sector No. 1 (6 o'clock position)	24	No DOP
Sector No. 2 (4 o'clock position)	24	$5 \cdot 10^{-2}$
Sector No. 3 (3 o'clock position)		pure anthranilic acid
Sector No. 4 (2 o'clock position)	48	No DOP
Sector No. 5 (12 o'clock position)	48	$5 \cdot 10^{-2}$
Sector No. 6 (11 o'clock position)		3-hydroxy-anthranilic acid
Sector No. 7 (9 o'clock position)	72	No DOP
Sector No. 8 (7 o'clock position)	72	$5 \cdot 10^{-2}$

It can be seen that in the 72 hours' incubation experiments the formation of fluorescent metabolites of tryptophan was considerable. The most characteristic difference between sectors 7 and 8 is a clearly fluorescent band in sector 8 due to anthranilic acid. The other fluorescent metabolites formed in 72 hours' experiments deserve more investigation. The authors noted that chromatograms of kynurenine and kynurenic acid did not correspond to these spots.

Anaerobic Tests. — Estimation of indole in the anaerobic tests gave the following results:

TABLE 1

	DOP concentration M/L	Indole formed M/L
Tube No. 1	—	$5.1 \cdot 10^{-1}$
Tube No. 2	$2.5 \cdot 10^{-2}$	$8.6 \cdot 10^{-1}$
Tube No. 3	$5.0 \cdot 10^{-2}$	$6.8 \cdot 10^{-1}$
Tube No. 4	$3.8 \cdot 10^{-2}$	$3.8 \cdot 10^{-1}$

A striking accumulation of indole is noted in the presence of small amounts of DOP. An increased accumulation is brought about also by addition of pyridoxine hydrochloride.

TABLE 2

	DOP Concentration M/L	Incubation Time	Indole Formed M/L
Tube No. 1	—	0 hours	$0.9 \cdot 10^{-1}$
Tube No. 2	—	16 hours	$4.3 \cdot 10^{-1}$
Tube No. 3	$2.5 \cdot 10^{-2}$	16 hours	$7.3 \cdot 10^{-1}$
Tube No. 4	$5.0 \cdot 10^{-2}$	16 hours	$7.7 \cdot 10^{-1}$
Tube No. 5	$7.5 \cdot 10^{-2}$	16 hours	$7.7 \cdot 10^{-1}$
Tube No. 6	$2.5 \cdot 10^{-1}$ pyridoxine HCl	16 hours	$11.1 \cdot 10^{-1}$
Tube No. 7	$5.0 \cdot 10^{-1}$ pyridoxine HCl	16 hours	$10.5 \cdot 10^{-1}$

DISCUSSION

In these experiments a striking effect of DOP on the metabolism of tryptophan in *e. coli* was noted. DOP is known to inhibit the activity of many enzymes dependent on pyridoxal phosphate. *In vivo*, however, an increased and not decreased accumulation of products of tryptophan metabolism was found, though the enzymes catalyzing the reactions leading to these products are known to be pyridoxal phosphate dependent. Anthranilic acid is an intermediate in the oxidation of tryptophan, and the increased accumulation of this intermediate might indicate that its further metabolism (see McCulloch) is most sensitive to inhibition by DOP. However, under anaerobic conditions an increased accumulation of indole was found, though indole is the terminal product of tryptophan metabolism under these conditions. An explanation of these effects might be found in an altered permeability for tryptophan and tryptophan metabolites in the presence of DOP.

SUMMARY

1. Deoxypyridoxine, $2.5 \times 10^{-2}M$, was found to increase the accumulation of anthranilic acid in the medium of tryptophan-oxidizing cells of *escherichia coli*.

2. Deoxypyridoxine, $2.5 \times 10^{-2}M$, was found to stimulate the anaerobic production of indole from tryptophan by *e. coli*.

3. These findings are discussed.

Acknowledgement. — The writers wish to thank Messrs Hoffmann, La Roche, Basle, for their kindness in supplying of a sample of 4-Deoxypyridoxine.

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NATURE OF LIPID INHIBITORS OF TICK-BORNE ENCEPHALITIS VIRUS HEMAGGLUTINATION

I

INHIBITORY LIPIDS OF HUMAN SERUM¹

by

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The presence of nonspecific inhibitors to arbor virus hemagglutinins in normal human and animal sera was established by Sabin and coworkers (13, 12, 2). They showed that these inhibitors are probably lipids. Recently, Porterfield and Rowe (7) have shown that isolated serum lipoproteins are inhibitory, but more definite information on the chemical nature of these nonspecific inhibitors of serum does not seem to be available.

Growing interest both in nonspecific inhibitors of the diphasic tick-borne encephalitis virus (TBEV) hemagglutination (14, 15) and in the lipids of human serum (11) prompted us to study the chemical nature of the TBEV hemagglutination inhibitors present in lipid extracts of normal human sera. This report describes results showing that the inhibitory activity of these extracts is due to certain mixtures of well-known serum lipid classes.

MATERIALS AND METHODS

The solvents used were of analytical grade. The adsorbents for chromatographic analyses were Mallinckrodt's silicic acid (analytical reagent, 100 mesh), the British Drug Houses' Kieselguhr (white), and Merck's aluminum oxide (standardized for chromatography according to Brockmann). They were used from intact bottles without any pretreatment.

¹ This work was aided by grants from the Sigrid Jusélius Foundation, Helsinki, and the Rockefeller Foundation.

*Extraction and Fractionation of Serum Lipids*¹. — The total lipid extract was prepared from fresh pooled, lyophilized human sera.² The extraction and purification method was essentially similar to that described by Folch *et al.* (5). Column chromatographic fractionation of the purified total lipid extract was carried out on silicic acid as described by Phillips (6). The nonphosphatides were then further fractionated on silicic acid, a stepwise gradient of chloroform in petroleum ether being used. This fractionation was completed by chromatography on aluminum oxide carried out according to the standard method of Reichstein *et al.* (8).

The eluted lipid fractions were evaporated under nitrogen at reduced pressure and temperatures not exceeding 50°C. The dry residues were immediately dissolved in nitrogen-saturated solvents and the solutions made up to definite volumes. Aliquots were analyzed gravimetrically, by the usual microanalytical methods of lipid chemistry¹, and by paper chromatography on silicic acid impregnated papers with known markers (9, 10).

Examination of Lipid Samples for Hemagglutination-Inhibiting Activity. — The solvents were evaporated from the lipid samples as described above. The dry residues were carefully suspended with a glass rod in a borate-saline-albumin solution of pH 9.0 (3). Serial twofold dilutions of the primary lipid suspensions were prepared with the above-mentioned solution as diluent.

The lipid suspensions were tested without previous erythrocyte absorption for TBEV hemagglutination-inhibiting activity according to the method described by Clarke and Casals (3), with the following specifications and modifications. The virus used was the strain «Belyanchikov» of the diphasic tick-borne encephalitis virus, kindly supplied by Prof. A. A. Smorodintsev, Leningrad. The antigen was prepared from infected mouse brains by the sucrose-acetone extraction method. The strength of the antigen was adjusted to contain 4—8 hemagglutinating units in 0.25 cc. Equal volumes (0.25 cc.) of the lipid suspensions and the antigen were incubated together at 4°C overnight. Rooster erythrocytes in a concentration of 0.20% were used as indicator. The settling of the erythrocytes took place at 4°C and pH 6.5. The results were read after 2—3 hours by examining the bottoms of the tubes.

Many of the inhibitory lipid suspensions agglutinated the erythrocytes even without added virus. But the titer of this lipid hemagglutination was lower than the inhibition titer, and therefore a zone of 3—8 tubes was seen where the erythrocytes were not agglutinated. The last tube of this zone was taken as a measure of the inhibitor content of the lipid suspensions.

Most of the noninhibitory lipids and lipid mixtures did not cause any lipid hemagglutination. A few noninhibitory lipids, which could agglutinate the red cells, did so only occasionally and always in low titers. Therefore, even with these lipids experiments could be carried out where the lipid hemagglutination was not observed.

¹ A report of our chemical work on serum lipids will be published later (11).

² The blood of donors in the fasting state was obtained from the Finnish Red Cross Blood Transfusion Service by courtesy of Dr. H. Nevanlinna, M.D.

RESULTS

Fractionation of Serum Lipids; Inhibitory Activity of Individual Fractions. — The chloroform phase of the Folch partition contained the «purified total lipids» which were inhibitory to TBEV hemagglutinin. The amount of inhibitors in these «purified total lipids» was about 30% of the original amount of inhibitors in the lyophilized serum. The aqueous phase of the Folch partition showed only traces of inhibitory activity.

The «purified total lipids» were fractionated on silicic acid. Data of this fractionation are given in Table 1.

TABLE 1
CHROMATOGRAPHY OF 6.2 GM. OF «PURIFIED TOTAL LIPIDS» FROM SERUM ON
100 GM. SILICIC ACID — KIESELGUHR (1:1; W/W)

Fraction No.	Eluting Solvent ¹	Amount of Nonvolatiles	Principal Components	Inhibitory Activity toward TBEV Hemagglutinin
1	C	4.35 Gm.	Nonphosphatides	+
2	C:M:W (400:100:6)	1.2 Gm.	Lecithins and «Cephalins» ²	—
3	C:M:W (400:100:6)	0.4 Gm.	Sphingomyelins	—
4	M:W (48:2)	0.25 Gm.	Lysolecithins	?

¹ C = Chloroform; M = Methanol; W = Water.

² «Cephalins» means here phosphatidyl ethanolamines and the poorly defined serum «inositides». Fraction 2 also contained the plasmalogens of serum.

Table 1 shows that only Fraction 1, which contained the serum nonphosphatides, had an inhibitory effect on TBEV hemagglutinin. The inhibitor content of Fraction 1 amounted to 5–10% of the original content of inhibitors in the «purified total lipids». Fractions 2 and 3, which contained the serum lecithins, phosphatidyl ethanolamines, «inositides», the corresponding plasmalogens and the sphingomyelins, were not inhibitory. Fraction 4, which contained the serum lysolecithins, caused hemolysis in our test system in tubes containing 25 μ g or more of this fraction. The more diluted tubes of this fraction were noninhibitory.

A sample of serum nonphosphatides corresponding to Fraction 1 in Table 1 was further fractionated on silicic acid. Data of this fractionation are given in Table 2.

TABLE 2
CHROMATOGRAPHY OF 3.3 GM. OF SERUM NONPHOSPHATIDES ON 100 GM. SILICIC
ACID — KIESELGUHR (1:1; w/w)

Fraction No.	Eluting Solvent ¹	Amount of Nonvolatiles	Principal Components	Inhibitory Activity toward TBEV Hemagglutinin
1	P:C (8:2)	1.92 Gm.	Cholesterol esters	—
2	P:C (1:1)	0.33 Gm.	Triglycerides	—
3	P:C (1:1)	0.98 Gm.	Triglycerides Free fatty acids	+
4	P:C (1:1)	0.05 Gm.	Free cholesterol Free cholesterol	—

¹ P=Petroleum ether; C=Chloroform.

Table 2 shows that only Fraction 3 had an inhibitory effect on TBEV hemagglutinin. Its activity was roughly equivalent to the whole inhibition induced by the original nonphosphatides. Fractions 1, 2 and 4, which contained pure cholesterol esters, triglycerides and free cholesterol, respectively, were not inhibitory.

A sample of Fraction 3 of Table 2 was then further fractionated on aluminum oxide. Data of this fractionation are given in Table 3.

TABLE 3
CHROMATOGRAPHY OF 203 MG. OF FRACTION 3 OF TABLE 2 ON 10 GM. ALUMINUM
OXIDE

Fraction No.	Eluting Solvent ¹	Amount of Nonvolatiles	Principal Components	Inhibitory Activity toward TBEV Hemagglutinin
1	P:E (15:1)	81 mg.	Triglycerides	—
2	P:E (9:1)	5 mg.	?	—
3	P:E (1:1)	82 mg.	Free cholesterol	—
4	E	5 mg.	?	—
5	C	Trace	?	—
6	C:M:W (1:5:2)	24 mg. ²	Free fatty acids	—?

¹ P=Petroleum ether; E=Ether; C=Chloroform; M=Methanol; W=Water.

² This figure gives the amount of lipids after elimination of contaminating column material.

None of the fractions shown in Table 3 had any inhibitory effect on TBEV hemagglutinin. Fraction 6, which contained the long chain fatty acids of serum, caused slight hemolysis in our

test system; its inhibitory character remained, therefore, somewhat obscure.

Now it seemed possible that the lipid inhibitor to TBEV hemagglutinin was either destroyed during the aluminum oxide chromatography or was a very small component irreversibly adsorbed onto the aluminum oxide. Another possibility was that the inhibitory activity of Fraction 3 of Table 2 was due to some definite mixture of lipids and not to any single substance. The last possibility was in fact shown to be true when the inhibitory activities of remixed fractions were studied.

Inhibitory Activity of Remixed Serum Lipid Fractions toward TBEV Hemagglutinin. — Samples of the six inactive fractions shown in Table 3 were remixed in their original proportions. Strikingly, the inhibitory activity of the lipid sample thus obtained toward TBEV hemagglutinin was identical with that of the original Fraction 3 of Table 2. This observation was simplified by tests showing that a recombination of only the three main fractions of Table 3 (Fractions 1, 2 and 6) also gave a fully active inhibitory preparation. These fractions represented pure triglycerides, free cholesterol and free fatty acids, respectively. Pairwise recombinations of these three substances were then tested for inhibitory activity toward TBEV hemagglutinin. The results are given in Table 4.

TABLE 4

INHIBITORY ACTIVITY TOWARD TBEV HEMAGGLUTININ OF BINARY MIXTURES OF SERUM TRIGLYCERIDES, CHOLESTEROL AND FREE FATTY ACIDS, WHICH WERE ISOLATED AS SHOWN IN TABLE 3 (The volume of the undiluted suspensions was 2.5 cc.)

Mixture	Inhibitory Titer
Triglycerides (8 mg.) + Cholesterol (8 mg.)	1:4
Triglycerides (8 mg.) + Fatty acids (2.4 mg.)	1:4
Cholesterol (8 mg.) + Fatty acids (2.4 mg.)	1:128
Fraction 3 of Table 2 (20 mg.)	1:256

Table 4 shows that the remixing of free cholesterol with free fatty acids of serum gave an active inhibitor to TBEV hemagglutinin. The mixtures of free cholesterol and triglycerides and those of free fatty acids and triglycerides were almost noninhibitory. Most

of the inhibitory activity of the serum nonphosphatides can therefore be ascribed to the combined effect of free cholesterol and free fatty acids of serum and not to any single substance alone.

The low yield of inhibitors of TBEV hemagglutination obtained in the nonphosphatide fraction after the first chromatographic fractionation (Table 1) led us now to make recombination experiments with fractions of this chromatogram too. The results of these experiments are summarized in Table 5.

TABLE 5

INHIBITORY ACTIVITY TOWARD TBEV HEMAGGLUTININ OF SOME MULTICOMPONENT SERUM LIPID MIXTURES (The volume of the undiluted suspensions was 2.5 cc.)

Mixture	Inhibitory Titer
Original «purified total lipids» of serum (10 mg.)	1:16
Nonphosphatides shown in Table 1 (7 mg.)	1:2
Mixture of	1:32
nonphosphatides shown in Table 1 (7 mg.)	
serum lecithins ¹ (1.9 mg.)	
sphingomyelins shown in Table 1 (0.6 mg.)	
lysolecithins shown in Table 1 (0.45 mg.)	

¹ This lecithin preparation is that shown in Table 1 after elimination of phosphatidyl ethanolamines and «inositides».

Table 5 shows that the whole inhibitory activity of the original unfractionated «purified total lipids» of serum can be reconstituted by simply mixing the weakly active nonphosphatide fraction and the totally inactive lecithin, sphingomyelin and lysolecithin fractions in the ratios in which they are present in the serum total lipids.

The results shown in Table 5 do not reveal whether the added phosphatides increase the inhibitory activity of the cholesterol-fatty acid mixture by some secondary effect, such as better emulsification, or by adding to the system a true potential inhibitor. Therefore the inhibitory activities of simple mixtures of free cholesterol and the individual serum phosphatides toward TBEV hemagglutinin were also measured. The results are given in Table 6. For comparison, similar data of our pure serum lipid fractions are also given in Table 6.

Table 6 shows that besides the free fatty acids the serum lecithins

TABLE 6

INHIBITORY ACTIVITY TOWARD TBEV HEMAGGLUTININ OF SOME PURE SERUM LIPIDS ALONE AND TOGETHER WITH ADDED CHOLESTEROL (The volume of the undiluted suspensions was 2.5 cc.)

Lipid Class	Added Cholesterol	Inhibitory Titer
Cholesterol esters (4 μ moles)	0	No inhibition
Triglycerides (4 μ moles)	0	No inhibition
Free cholesterol (10 μ moles)	0	No inhibition
Free fatty acids (4 μ moles)	0	No inhibition
Lecithins ¹ (4 μ moles)	0	No inhibition
Sphingomyelins (4 μ moles)	0	No inhibition
Lysolecithins (4 μ moles)	0	?
Cholesterol esters (4 μ moles)	10 μ moles	No inhibition
Triglycerides (4 μ moles)	10 μ moles	1:2
Free fatty acids (4 μ moles)	10 μ moles	1:64
Lecithins ^{1, 2} (4 μ moles)	10 μ moles	1:64
Sphingomyelins (4 μ moles)	10 μ moles	1:32
Lysolecithins (4 μ moles)	10 μ moles	1:64

¹ This lecithin preparation was free of phosphatidyl ethanolamines and of «inositides».

² The behaviour of cholesterol — lecithin mixtures, at least, depends greatly on the relative proportions of the two components.

thins ¹, sphingomyelins and lysolecithins are also strong inhibitors of TBEV hemagglutination when admixed with free cholesterol. Their activities as compared on molar basis are rather similar. All these lipids were noninhibitory to TBEV hemagglutinin without added cholesterol. Table 6 also shows that the cholesterol esters and the triglycerides of serum do not act as inhibitors to TBEV hemagglutinin, either alone or when admixed with free cholesterol.

DISCUSSION

The results presented show that normal human serum contains no single lipid or lipid class which we could find capable of inhibiting TBEV hemagglutination. The distinct inhibitory properties of lipid extracts from normal serum are due to mixtures of lipids. These serum lipid mixtures are inhibitory to TBEV hemagglutinin when they contain free cholesterol and any of the lipids which

¹ The serum phosphatidyl ethanolamines and the serum «inositides» were needed for other purposes (11).

we would like to call »potential inhibitors». These »potential inhibitors» include free fatty acids, lecithins, sphingomyelins and lysolecithins of serum. In a very recent paper, Porterfield and Rowe (7) have reported results from which they conclude that the inhibitory activity of lipid extracts from red cells toward hemagglutination by several arbor Group B viruses is possibly due to traces of an unknown phosphatide hidden in the sphingomyelin and in other undefined phosphatides. We think that they have overlooked the possibility of lipid mixtures as inhibitors to hemagglutination by Group B arbor viruses.

Both cholesterol and the phospholipids are known to be »bound» in lipoproteins in native serum. But more than half of the surface of low density lipoproteins is thought to be of lipid nature, and also in high density lipoproteins at least the polar parts of the lipids are believed to be exposed to aqueous environment. Therefore we think it possible that structures similar to those in our lipid suspensions are also responsible for the inhibitory property of the whole serum. The free fatty acids possibly constitute an exception, because, being mostly albumin-bound, they are probably not in close contact with cholesterol in the serum.

In an earlier paper from our laboratory it has been shown that TBEV hemagglutination-inhibiting extracts can be obtained from erythrocytes with fat solvents (15). The chemical basis of this finding can now be explained, as it is known that free cholesterol, lecithin and also sphingomyelin are major lipid components of red cells. They are, in fact, believed to form part of the surface of erythrocytes (4), and therefore they can be thought capable of forming the virus receptors.

The experiments described in this report were carried out with a single virus strain belonging to Casals' Group B of the arthropod-borne viruses (1). But it is known that normal human sera contain nonspecific inhibitors to hemagglutinins of many arbor viruses. Furthermore these inhibitors can be removed from sera by similar methods, *e.g.*, by acetone extraction or kaolin adsorption (3). Therefore we believe that our observations concerning the lipid inhibitors to one special Group B arbor virus hemagglutinin are probably valid for related viruses too.

SUMMARY

A purified total lipid extract of normal human serum inhibited hemagglutination by the diphasic tick-borne encephalitis virus. This total lipid extract was fractionated by column chromatography. It was found that the inhibitory activity was due to mixtures of free cholesterol and the principal phosphatides of serum (lecithins, sphingomyelins and lysolecithins), and to mixtures of free cholesterol and free fatty acids.

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NATURE OF LIPID INHIBITORS OF TICK-BORNE ENCEPHALITIS VIRUS HEMAGGLUTINATION

II

INHIBITORY ACTIVITY OF AUTHENTIC LIPIDS AND THEIR MIXTURES ¹

by

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In a previous report (2) we have shown that the inhibitory activity of serum lipid extracts toward diphasic tick-borne encephalitis virus (TBEV) hemagglutinin was due to certain mixtures of lipids, whereas no single lipid or lipid class of serum was found capable in itself of inhibiting TBEV hemagglutination. The serum lipid mixtures were inhibitory when they contained free cholesterol and any of the several lipids which we have called »potential inhibitors». These »potential inhibitors» included the lecithins, sphingomyelins, lysolecithins and free fatty acids of serum.

This report describes assays of inhibitory activities toward TBEV hemagglutinin of authentic pure lipid samples and their mixtures. The results allow some suggestions to be made concerning the essential structural features of the lipid inhibitors of TBEV hemagglutination.

MATERIALS AND METHODS

The source and quality of the lipid samples tested are listed below:
Acetic and butyric acids: Puriss. (E. Merck, Germany)

¹ This work was aided by grants from the Sigrid Jusélius Foundation, Helsinki, and the Rockefeller Foundation.

Caproic acid: (The Coleman & Bell Co., U.S.A.)

All other fatty acids: Puriss. (Fluka, Switzerland)

Methyl palmitate: (Armour Research Division, U.S.A.)

Monopalmitin: (Eastman Organic Chemicals, U.S.A.)

Trilinolein: (The Hormel Institute, Austin, U.S.A.)

Trimyristin: (C. A. F. Kahlbaum, Germany)

3,9-Diethyl-tridecanol-6-sulphate-Na: (Fluka, Switzerland)

Dodecanol-sulphate-Na: (University Drugstore, Helsinki)

Lithocholic acid: (The Mann Fine Chemicals, U.S.A.)

Desoxycholic and cholic acids: (S. A. F. Hoffmann-La Roche, Switzerland)

Phosphatidyl ethanolamine: Synth., L- α -dipalmitoyl-, Puriss. (Fluka, Switzerland)

Phosphatidyl choline: Synth., L- α -dipalmitoyl-, Puriss. (Fluka, Switzerland)

Phosphatidyl inositol: From wheat germs (Dr. M. Faure, Institut Pasteur, Paris)

Cardiolipin: From ox heart (Dr. M. McFarlane, Lister Institute of Preventive Medicine, London)

Cholesterol: (The Coleman & Bell Co., U.S.A.)

Cholesterol stearate: (The British Drug Houses, England)

Sitosterol, stigmasterol and ergosterol: (S. A. F. Hoffmann-La Roche, Switzerland)

The lipid samples were dissolved in organic solvents. After the solvents were evaporated, the lipids were suspended and examined for hemagglutination-inhibiting activity as described previously (2).

Some of the lipids and lipid mixtures agglutinated the test erythrocytes without added virus. This lipid hemagglutination was generally only of low titer, the inhibition induced by the hemagglutinating lipids appearing as a zone. On this account, suspensions of the hemagglutinating »noninhibitory« lipids could only be studied reliably at greater dilutions which no longer caused agglutination of the test erythrocytes.

RESULTS

Inhibitory Activity of Pure Lipid Samples. — Results of inhibition assays of pure lipid samples are presented in Table 1.

Table 1 shows that the pure fatty acids and their esters, bile acids, and sterols and their esters investigated were noninhibitory to TBEV hemagglutinin in 1.6 millimolar concentrations. The common glycerophosphatides and two sulphuric acid esters of long chain alcohols could not be shown to be inhibitory either, but owing to hemagglutination caused by these lipids the inhibition assays were reliable only under 0.2–0.4 millimolar concentrations. The only lipid alone capable of inhibiting TBEV hemagglutination was cardiolipin.

TABLE 1
INHIBITORY ACTIVITY OF PURE LIPIDS TOWARD TBEV HEMAGGLUTININ
(The volume of the undiluted suspensions was 2.5 cc.)

Lipid	Inhibitory Titer
<i>Saturated Fatty Acids:</i>	
Acetic acid (4 μ moles)	0
Butyric acid "	0
Caproic acid "	0
Caprylic acid "	0
Capric acid "	0
Lauric acid "	0
Myristic acid "	0
Palmitic acid "	0
Stearic acid "	0
Arachidic acid "	0
Behenic acid "	0
Lignoceric acid "	0
Cerotic acid "	0
<i>Unsaturated C₁₈ Fatty Acids:</i>	
Oleic acid (4 μ moles)	0
Linoleic acid "	0
Linolenic acid "	0
<i>Fatty Acid Esters:</i>	
Methyl palmitate (4 μ moles)	0
Monopalmitin "	0
Trilinolein "	0
Trimyristin "	0
<i>Sulphuric Acid Esters of Long Chain Alcohols:</i>	
3,9-diethyl-tridecanol-6-sulphate-Na (4 μ moles)	0 (?) ¹
Dodecanol-sulphate-Na (4 μ moles)	0 (?) ¹
<i>Bile Acids:</i>	
Lithocholic acid (4 μ moles)	0
Desoxycholic acid "	0
Cholic acid "	0
<i>Phosphatides:</i>	
Phosphatidyl ethanolamine (4 μ moles)	0
Phosphatidyl choline "	0 (?) ¹
Phosphatidyl inositol "	? ²
Cardiolipin (2 μ moles)	1: 256
<i>Sterols:</i>	
Cholesterol (10 μ moles)	0
Sitosterol "	0
Stigmasterol "	0
Ergosterol "	0 (?) ¹
Cholesterol stearate "	0

¹ Because of hemagglutination caused by this lipid, the inhibition assay could be reliably carried out only after 4-8-fold primary dilution.

² Because of strong hemagglutination caused by this lipid, the inhibition assay could not be carried out.

Inhibitory Activity of Pure Lipids Admixed with Cholesterol. — Results of inhibition assays of even numbered, saturated straight chain fatty acids admixed with cholesterol are presented in Table 2.

TABLE 2
INHIBITORY ACTIVITY TOWARD TBEV HEMAGGLUTININ OF EVEN NUMBERED, SATURATED FATTY ACIDS ADMIXED WITH CHOLESTEROL
(The volume of the undiluted suspensions was 2.5 cc.)

Fatty Acid			Added Cholesterol	Inhibitory Titer
Name	Chain Length	Amount		
Acetic acid	C ₂	4 μ moles	10 μ moles	0
Butyric acid	C ₄	"	"	0
Caproic acid	C ₆	"	"	0
Caprylic acid	C ₈	"	"	0
Capric acid	C ₁₀	"	"	1: 1
Lauric acid	C ₁₂	"	"	1: 16
Myristic acid	C ₁₄	"	"	1: 32
Palmitic acid	C ₁₆	"	"	1: 32
Stearic acid	C ₁₈	"	"	1: 32
Arachidinic acid	C ₂₀	"	"	1: 32
Behenic acid	C ₂₂	"	"	1: 16
Lignoceric acid	C ₂₄	"	"	1: 1
Cerotic acid	C ₂₆	"	"	0

Table 2 shows that only fatty acids in the chain length range of C₁₂—C₂₂ were »potential inhibitors» of TBEV hemagglutination.

A comparison of the inhibitory activities of stearic, oleic, linoleic and linolenic acids admixed with cholesterol is presented in Table 3. It is seen that the number of double bonds in C₁₈ fatty acids had little or no effect on their potential inhibitory activity.

TABLE 3
INHIBITORY ACTIVITY TOWARD TBEV HEMAGGLUTININ OF STEARIC, OLEIC, LINOLEIC AND LINOLENIC ACIDS ADMIXED WITH CHOLESTEROL
(The volume of the undiluted suspensions was 2.5 cc.)

Fatty Acid				Added Cholesterol	Inhibitory Titer
Name	Chain Length	No. of Double Bonds	Amount		
Stearic acid	C ₁₈	0	4 μ moles	10 μ moles	1: 16
Oleic acid	C ₁₈	1	"	"	1: 32
Linoleic acid	C ₁₈	2	"	"	1: 32
Linolenic acid	C ₁₈	3	"	"	1: 32

Inhibition assays of mixtures containing varying amounts of cholesterol and palmitic acid are presented in Table 4.

TABLE 4

INHIBITORY ACTIVITY TOWARD TBEV HEMAGGLUTININ OF MIXTURES CONTAINING VARYING AMOUNTS OF PALMITIC ACID AND CHOLESTEROL
(The volume of the undiluted suspensions was 2.5 cc.)

Palmitic Acid	Added Cholesterol	Inhibitory Titer
0.25 μ moles	10 μ moles	1: 1
1 "	"	1: 8
4 "	"	1: 32
8 "	"	1: 64
32 "	"	1: 64
128 "	"	1: 64

Table 4 shows that with a given amount of cholesterol the inhibitory activity increased with increasing amounts of palmitic acid until a plateau was reached. The results in Table 4 can be recalculated to show that with a given amount of palmitic acid the inhibitory activity increases with increasing amounts of cholesterol until here, too, a plateau is reached. Mixtures of oleic acid and cholesterol behaved similarly, but more oleic acid had to be added to cholesterol before the plateau was reached. The inhibitory activity of mixtures containing maximal amounts of oleic acid was 2—4 times higher than that of the corresponding cholesterol — palmitic acid mixtures. Differences of this kind are probably related to the internal geometric structures of the cholesterol — fatty acid particles in the lipid suspensions.

Comparison of the inhibitory activities of free palmitic acid and its methyl ester, both admixed with cholesterol, is shown in Table 5. It is seen that to exert inhibitory activity the acid must

TABLE 5

COMPARISON OF INHIBITORY ACTIVITY TOWARD TBEV HEMAGGLUTININ OF PALMITIC ACID AND METHYL PALMITATE BOTH ADMIXED WITH CHOLESTEROL
(The volume of the undiluted suspensions was 2.5 cc.)

Lipid	Added Cholesterol	Inhibitory Titer
Palmitic acid (4 μ moles)	10 μ moles	1: 32
Methyl palmitate "	"	0

be in free form. In conformity with Table 5 and with our previous work on the serum lipids, mixtures of cholesterol with trilinolein, trimyristin and α -monopalmitin were also found to be totally non-inhibitory to TBEV hemagglutinin.

Table 6 shows that sulphuric acid esters of long chain alcohols formed equally active inhibitors as fatty acids of similar chain lengths, when admixed with cholesterol.

TABLE 6

COMPARISON OF INHIBITORY ACTIVITY TOWARD TBEV HEMAGGLUTININ OF TWO SULPHURIC ACID ESTERS OF LONG CHAIN ALCOHOLS, AND OF TWO FATTY ACIDS OF SIMILAR CHAIN LENGTHS

(The volume of the undiluted suspensions was 2.5 cc.)

Lipid	Added Cholesterol	Inhibitory Titer
3,9-diethyl-tridecanol-6-sulphate-Na (4 μ moles)	10 μ moles	1: 32
Dodecanol-sulphate-Na "	"	1: 16
Stearic acid "	"	1: 16
Myristic acid "	"	1: 32

The inhibitory activities of some cholesterol — bile acid mixtures are given in Table 7. It is seen that the three bile acids, which in themselves were noninhibitory, also formed inhibitors when admixed with cholesterol. Their inhibitory activity was, however, only $\frac{1}{4}$ of that of fatty acid — cholesterol mixtures, as compared on molar basis.

TABLE 7

INHIBITORY ACTIVITY TOWARD TBEV HEMAGGLUTININ OF THREE COMMON BILE ACIDS ADMIXED WITH CHOLESTEROL

(The volume of the undiluted suspensions was 2.5 cc.)

Bile Acid	Added Cholesterol	Inhibitory Titer
Lithocholic acid (4 μ moles) 	10 μ moles	1: 2
Desoxycholic acid " 	"	1: 8
Cholic acid " 	"	1: 4

The inhibitory activities of glycerophosphatides admixed with cholesterol are given in Table 8. All the phosphatide — cholesterol mixtures tested were inhibitory and their inhibitory activity was of the same degree as that of fatty acid — cholesterol mixtures, as

compared on molar basis. The only exception was found in cholesterol — cardiolipin mixtures, which were about 8 times more active than mixtures of the other phosphatides and cholesterol. However, it should be noted that cholesterol was without effect on the inhibitory activity of cardiolipin which even alone was an active inhibitor.

TABLE 8
INHIBITORY ACTIVITY TOWARD TBEV HEMAGGLUTININ OF SOME PHOSPHATIDES
ADMIXED WITH CHOLESTEROL
(The volume of the undiluted suspensions was 2.5 cc.)

Phosphatide	Added Cholesterol	Inhibitory Titer
Phosphatidyl ethanolamine (4 μ moles)	10 μ moles	1: 16
Phosphatidyl choline "	"	1: 64
Phosphatidyl inositol "	"	1: 64
Cardiolipin "	"	1: 256

Inhibitory Activity of Palmitic Acid Admixed with Different Sterols. — Results of inhibition assays of palmitic acid admixed with a few sterols are given in Table 9. Table 9 shows that besides cholesterol only sitosterol could activate palmitic acid to form an inhibitor of TBEV hemagglutination.

TABLE 9
INHIBITORY ACTIVITY TOWARD TBEV HEMAGGLUTININ OF SOME STEROLS ADMIXED WITH PALMITIC ACID
(The volume of the undiluted suspensions was 2.5 cc.)

Sterol	Added Palmitic Acid	Inhibitory Titer
Cholesterol (10 μ moles) 	4 μ moles	1: 32
Sitosterol " 	"	1: 16
Stigmasterol " 	"	0 (?) ¹
Ergosterol " 	"	0 (?) ¹
Cholesterol stearate " 	"	0

¹ Because of hemagglutination caused by this lipid mixture, the inhibition assay could be reliably carried out only after 4—8-fold primary dilution.

DISCUSSION

The results presented fully confirm our previous findings with the lipids of human serum (2). With the exception of cardiolipin, we have not as yet found any pure lipid capable of inhibiting TBEV hemagglutination at a concentration around $1.6 \mu\text{moles per cc.}$ Some reservation to this statement must be made, however, since a few lipids agglutinated the red cells at this concentration and could be reliably studied only in suspensions 4—8 times more dilute. On the other hand, several different lipids could be shown to have an inhibitory effect on TBEV hemagglutinin when admixed with free cholesterol. Our results suggest that acidic groups probably play an essential role in the activity of these «potential inhibitors». The role of cholesterol could be explained by assuming that both the hydroxyl groups of cholesterol molecules and the acidic groups of the «potential inhibitors» are needed to bind the virus to the inhibitor particles. This hypothesis does not, however, explain the noninhibitory character of such mixtures as palmitic acid and stigmasterol.

In an earlier paper from our laboratory it was suggested that the binding between TBEV and red cells may be electrostatic in nature (1). If this is true and if the lipid inhibitors described are analogous to the receptores on erythrocyte surfaces, it seems that the binding sites of the virus are of base character.

The inhibitory power of the cholesterol — fatty acid mixtures, for instance, makes it tempting to speculate about the use of such mixtures as adsorbents for the purification of the virus. Such a procedure might well be successful, since earlier work in our laboratory has shown that adsorption of TBEV on erythrocytes and subsequent elution of the virus can be effectively controlled simply with proper adjustment of the pH value of the surrounding fluid (1).

SUMMARY

Suspensions of pure fatty acids and their esters, bile acids, sulphuric acid esters of long chain alcohols, and the common glycerophosphatides were found to be noninhibitory to diphasic tick-borne encephalitis virus hemagglutinin when studied in 0.2—1.6 millimolar concentrations.

All these lipids, with the exception of free fatty acids with very short or very long chains and neutral esters of fatty acids, were inhibitory when admixed with free cholesterol.

Besides cholesterol, sitosterol could also activate palmitic acid to form inhibitors, but stigmasterol, ergosterol and cholesterol stearate were without effect.

Cardiolipin was inhibitory to tick-borne encephalitis virus hemagglutinin even alone.

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REPORT ON ENTEROVIRUS ISOLATIONS IN
THE STATE SERUM INSTITUTE IN FINLAND

(Sept. 1959—March 1960)

by

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(Received for publication October 4, 1960)

This paper presents the results of the routine diagnosis of enteroviruses using the tissue culture technique in the State Serum Institute in Finland from September 1959 to March 1960. In the same period, the reported cases of paralytic poliomyelitis were 243 (302 cases in the year 1959, it is 6,9/100'000).

The material studied derived from patients with symptoms indicating a possible viral disease. All specimens were sent from hospitals or by private physicians on a routine basis. During the mentioned period, the State Serum Institute received 802 pharyngeal and 440 fecal specimens. All specimens were studied by means of the Hela cell and the primary amnion cell culture technique described previously (1).

From the pharyngeal material only one enterovirus ECHO 9, was isolated from one specimen.

440 fecal samples represented 366 patients. From them 144 virus strains were isolated, mostly of Polio Type I (115 strains). 3 strains of ECHO 6, 8 strains of ECHO 9, 2 strains of ECHO 11, 3 strains of Adeno 7 and 13 still untyped strains were also isolated. They are described in the following table.

	Reported Cases according to Clinical Diagnosis	Isolated Virus Strains					
		Polio 1	Echo 6	Echo 9	Echo 11	Adeno 7	Untyped
Paralytic cases	108	82	—	—	1	—	2
Meningitis *	102	30	—	6	—	—	8
Infectio acuta	39	—	—	1	—	—	1
Pro obs	15	3	—	—	1	—	—
Other cases	102	—	3	1	—	3	2
	366	115	3	8	2	3	13

The table shows the reported cases according to the clinical diagnosis and the isolated virus strains. Out of 108 reported paralytic cases, 82 isolated Polio Type I strains make 76%.

During the period in question Polio Type I was the most common virus. ECHO 6 was isolated from three children only, living in a suburb of Helsinki. They were all suffering from jaundice and two of them were under treatment in the children's hospital under a diagnosis: Hepatitis epidemica. ECHO 6 was not isolated from any other specimen. ECHO 9-viruses were perhaps residues of an ECHO 9-epidemic of the previous year (2). Adeno 7 virus was circulating in Finland also in the spring of 1959.

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DENSITY OF HUMAN CARPAL, METACARPAL AND DIGITAL BONES¹

by

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ANTTI TELKKÄ

(Received for publication October 5, 1960)

The mineral content of the human skeleton is not uniform, and there are considerable differences in the densities of various bones according to their functional demands (2, 3, 4). This fact is interesting from several points of view. Apparently, it makes it impossible to predict the mineralization grade of the whole skeleton based on measurements made on one bone or on a few bones only. On the other hand, there certainly is some regularity in the variation of the bone density in various parts of the skeleton. These variations can be individual but it would be very valuable to find out some general rules in this respect, because it would render it possible to continue the work with the aid of some of the numerous existing roentgenological methods in living material. So far, the materials examined have been very limited. Our purpose was in this work to increase the knowledge regarding variations of the densities of bones which are easily accessible by means of the roentgenological methods, by examining systematically the small bones of the hand.

MATERIAL AND METHODS

The material examined consisted of the carpal, metacarpal and digital bones of five male and five female individuals aged 26—74. The bones were dried in an oven at 70°C for eight days. They were

¹ Aided by a grant from The Sigrid Jusélius Foundation.

weighed with a Mettler balance and then dipped in a perspex glue for coating them with a thin perspex-film. Their volumes were measured employing specially constructed pycnometers. In its details, the method has been described in our previous paper (2).

RESULTS

The results obtained (Table 1) were analyzed by the use of standard methods of statistical analysis (1) from several points of view.

There was a statistically significant difference between both sexes in all bones examined. The mean density of the bones of the male group was higher than that of the female group. The scatter was more pronounced in the female group. Therefore, from the single bones investigated, in the bones of the wrist and in those of the thumb, the difference between the sexes was significant as such one ($P < 0.05$).

The difference between the densities of the bones of right and left side was less pronounced than in the material examined by Virtama (4). This probably is partially dependent in the difference of the methods used; in the paper mentioned the bones were burned and their ash contents were measured.

In the present paper, the most marked differences between the right and left side were as follows:

- the distal phalanx of the third finger of the right side was denser than that of the left side ($P < 0.05$).
- in one of the cases examined all the bones of the right hand were denser than those of the left hand ($P < 0.001$).

The lunate was most dense of the wrist bones of the male group. The difference was significant as compared with other bones of the wrist except for the scaphoid. This bone was very nearly of the same density range as the lunate. In the female group, the scaphoid was the densest one, then the greater multangular and the lunate ($P < 0.02$). Between these bones there were no significant differences. The lesser multangular and the pisiform were less dense than the other wrist bones in both sexes ($P < 0.01$), and there was no significant difference between the densities of these two bones.

The metacarpals were less dense than the bones of the fingers

TABLE 1
BONE DENSITIES IN THE MALE AND FEMALE GROUPS

Bone	Male		Female		P
	Mean	S. D.	Mean	S. D.	
Scaphoid	0.824	0.023	0.700	0.043	0.05
Greater Multangular ..	0.786	0.095	0.678	0.092	0.05
Lesser Multangular ..	0.644	0.072	0.524	0.168	0.05
Capitate	0.760	0.043	0.599	0.096	0.001
Hamate	0.785	0.079	0.644	0.152	0.05
Triquetral	0.803	0.059	0.649	0.115	0.001
Pisiform	0.650	0.057	0.579	0.104	0.05
Lunate	0.847	0.069	0.676	0.122	0.01
1 st Metacarpal	0.770	0.025	0.660	0.146	0.05
Thumb, 1 st Phalanx ..	0.933	0.017	0.756	0.151	0.001
Thumb, 2 nd Phalanx ..	0.952	0.068	0.750	0.200	0.05
2 nd Metacarpal	0.879	0.023	0.810	0.173	The difference is significant in no single case.
Index, 1 st Phalanx ..	1.008	0.050	0.894	0.208	
Index, 2 nd Phalanx ..	1.056	0.057	0.929	0.209	In all cases the mean is greater on the male side, however, which gives after Sign-test male-female difference at the level $P < 0.001$
Index, 3 rd Phalanx ..	0.972	0.106	0.964	0.190	
3 rd Metacarpal	0.893	0.046	0.811	0.199	
Medius, 1 st Phalanx ..	0.988	0.011	0.899	0.073	
Medius, 2 nd Phalanx ..	1.071	0.051	1.031	0.276	
Medius, 3 rd Phalanx ..	0.965	0.083	0.910	0.234	
4 th Metacarpal	0.840	0.037	0.769	0.176	
Annularis, 1 st Phalanx	1.003	0.061	0.875	0.255	
Annularis, 2 nd Phalanx	1.084	0.091	0.994	0.248	
Annularis, 3 rd Phalanx	1.048	0.068	0.977	0.255	
5 th Metacarpal	0.817	0.027	0.754	0.184	
Minimus, 1 st Phalanx	0.952	0.167	0.883	0.230	
Minimus, 2 nd Phalanx	1.031	0.087	0.926	0.235	
Minimus, 3 rd Phalanx	1.082	0.086	0.944	0.251	

($P < 0.01$). The first metacarpal was less dense ($P < 0.01$) than the others. The second and third metacarpals were the densest bones of this group ($P < 0.05$), and there were no significant difference between these two bones. These two metacarpals were also denser than the lunate and the greater multangular ($P < 0.01$).

The proximal phalanges were less dense than other phalanges ($P < 0.05$). There were no significant differences between the intermediate and distal phalanges of the fingers. The proximal phalanx of the thumb was less dense than the other proximal phalanges, the densest of which were the second, third and the

fourth proximal phalanges ($P < 0.01$). There were no significant differences in the density of the intermediate and distal phalanges as compared with each other.

DISCUSSION

The present results are in agreement with those obtained previously with the aid of the ash weight method (4). The material examined in this work was complete consisting of all small bones of the hand. It was possible to find out some general rules in the density variations, *e.g.* the lunate and the scaphoid are the densest of the wrist bones which, again, are less dense than the densest metacarpals or phalanges. The variation is, however, rather irregular in individual cases, and it seems to be hazardous to draw any definite conclusions on the other bones based on the density values of one bone examined. On the other hand, if the densities of three different type of the bones, say, the density of one of the phalanges, that of one of the metacarpals and that of one of the wrist bones are known, it seems to be possible to evaluate the mineralization grade of all the small hand bones with a fair dependability. The density of the bones of opposite side is, again, in some cases considerably different and therefore, the measurements should be made on both sides.

The correlation of the bone density of the extremities to that of the vertebral column or to other parts of the skeleton is a further problem to be examined, but according to the results of Trotter and her associates (3) it seems not to be justified to draw conclusions on the latter based on the measurements on the former bones. However, there may be some general rules valid in most of the cases, as there is between the densities of the small bones of the extremities. An investigation of the densities of the bones of a living material with some of the radiological methods presented would certainly give more information concerning the influence of the functional demands on the densities of individual bones.

SUMMARY

The density of the carpal, metacarpal and finger bones of five male and five female individuals was investigated. The bones of the male group were denser than those of the female group. The lunate

and the scaphoid are the densest of the wrist bones, which, again, are less dense than the densest metacarpals, second and third, and the finger bones. The proximal phalanges were less dense of the finger bones. If the density of one wrist bone, one metacarpal and one phalanx is known, it seems to be possible to evaluate the mineralization grade of all the small hand bones with reasonable accuracy.

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